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PREPARATION OF *d*(—)-GLUTAMIC ACID FROM *dl*-GLUTAMIC ACID BY ENZYMATIC RESOLUTION

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It has previously been shown (1) that papain as well as other proteinases can perform the enzymatic synthesis of CO—NH linkages. Thus, in the presence of HCN-papain, carbobenzoxyglycine and aniline were combined to form carbobenzoxyglycine anilide. When a derivative of an asymmetric amino acid was employed, only the derivative of the *l*-amino acid participated in the synthesis. The asymmetric course of this reaction provides the basis for a general method for the resolution of racemic amino acids. In this note the preparation of the "unnatural" *d*(—)-glutamic acid is described. This amino acid has been employed for the synthesis of peptides required in studies on the antipodal specificity of proteolytic enzymes of normal and pathological tissues.

Carbobenzoxy-*dl*-glutamic acid was treated with aniline in the presence of papain-cysteine. The filtrate from the carbobenzoxy-*l*-glutamic acid anilide usually yielded a mixture of carbobenzoxy-*d*- and *l*-glutamic acids in the proportion of 4:1. On hydrogenation and conversion of the glutamic acids to the hydrochlorides, it was possible to obtain, after a few recrystallizations (2), pure *d*(—)-glutamic acid.

Resolutions of *dl*-glutamic acid have been described by Schulze and Bosshard (3), Fischer (4), and Ehrlich (5). Recently Ivanovics and Bruckner (6) have isolated *d*(—)-glutamic acid from the capsular substance of bacteria of the *mesentericus* group.

EXPERIMENTAL

dl-Glutamic Acid Hydrochloride—This substance was prepared according to the method described in an earlier paper (7).

Carbobenzoxy-dl-Glutamic Acid—This compound was prepared as described for the *l* form (8). 65 gm. of *dl*-glutamic acid hydrochloride yielded 81 gm. of carbobenzoxy-*dl*-glutamic acid. M.p., 119°.

$C_{13}H_{15}O_6N$.	Calculated.	C 55.5, H 5.4, N 5.0
281.1	Found.	" 55.4, " 5.4, " 5.2

Resolution of Carbobenzoxy-dl-Glutamic Acid with Papain-Cysteine—70 gm. of carbobenzoxy-*dl*-glutamic acid were dissolved in 135 cc. of 2 N NaOH and added to 47 gm. of aniline. A solution of 3 gm. of cysteine hydrochloride in 100 cc. was added, followed by the enzyme solution containing 8 gm. of purified papain (9) in 200 cc. of water. 200 cc. of 0.2 M citrate buffer (pH 5.0) were added and the reaction mixture was made up to 1 liter. Crystallization of the carbobenzoxy-*l*-glutamic acid anilide began almost instantly. The reaction mixture was left at 40° for 2 days. The crystalline precipitate was filtered off, and the filter cake resuspended in 600 cc. of water and refiltered. All the filtrates and washings were combined and the clear solution was concentrated to about 200 cc. Concentrated hydrochloric acid was added (Congo red acidity) and the resulting syrup extracted three times with ethyl acetate. The ethyl acetate layer was washed with water, dried, and concentrated to a syrup. Petroleum ether was added; and, after the material had stood overnight in the cold, the syrup crystallized. The air-dried material weighed 38 gm. $[\alpha]_D^{23} = +4.1°$ (8.4 per cent in glacial acetic acid). The specific rotation of carbobenzoxy-*l*-glutamic acid is $-7.1°$ (8).

d-Glutamic Acid Hydrochloride—35 gm. of the above mixture of carbobenzoxy-*d*- and *l*-glutamic acids were dissolved in 100 cc. of methyl alcohol and hydrogenated with palladium black as the catalyst. The hydrogenation required 8 to 10 hours and the glutamic acid separated out. Water was added and the catalyst was filtered off and washed with hot water. The combined filtrates were evaporated down, yielding a crystalline residue. This material was dissolved in 70 cc. of 20 per cent hydrochloric acid, and dry HCl passed through the solution. The crystals that separated out on standing in the ice box were collected the next day and dried *in vacuo* over KOH. Yield, 16 gm. $[\alpha]_D^{23} = -23.0°$ (3.4 per cent in 10 per cent hydrochloric acid).

Two recrystallizations from 20 per cent hydrochloric acid raised the rotation to the correct value. Yield, 11.8 gm., or 46 per cent of the theory (based on the quantity of *dl*-glutamic acid hydrochloride employed). $[\alpha]_D^{23} = -32.0°$ (3.6 per cent in 10 per cent hydrochloric acid).

$C_5H_9O_4N \cdot HCl$.	Calculated.	C 32.7, H 5.5, N 7.6
183.6	Found.	" 32.9, " 5.4, " 7.5

BIBLIOGRAPHY

1. Bergmann, M., and Fraenkel-Conrat, H., *J. Biol. Chem.*, **119**, 707 (1937) Bergmann, M., *Chem. Rev.*, **22**, 423 (1938).
2. Graff, S., *J. Biol. Chem.*, **130**, 13 (1939).
3. Schulze, E., and Bosshard, E., *Z. physiol. Chem.*, **10**, 138 (1886).
4. Fischer, E., *Ber. chem. Ges.*, **32**, 2451 (1899).
5. Ehrlich, F., *Biochem. Z.*, **63**, 379 (1914).
6. Ivanovics, C., and Bruckner, V., *Z. physiol. Chem.*, **247**, 281 (1937).
7. Bergmann, M., and Zervas, L., *Biochem. Z.*, **203**, 280 (1928).
8. Bergmann, M., and Zervas, L., *Ber. chem. Ges.*, **65**, 1192 (1932).
9. Grassmann, W., *Biochem. Z.*, **279**, 131 (1935).

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THE SPECIFICITY OF CARBOXYPEPTIDASE

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All the information hitherto available with respect to the specificity of carboxypeptidase has been obtained by the use of crude enzyme preparations (1). Consequently, there still remains some uncertainty as to whether the previously reported hydrolyses of various synthetic substrates, attributed to the action of carboxypeptidase, are all due to the same enzyme. Indeed, Abderhalden and Abderhalden (2) have advanced the hypothesis that the hydrolysis of chloroacetyltyrosine and of similar halogen-acylated amino acids and peptides is attributable to an acylase different from the genuine carboxypeptidase. The latter is assumed to attack only those polypeptides in which the amino group is masked by a benzoyl or naphthalenesulfonyl group.

The specificity of carboxypeptidase has now been reinvestigated by means of crystalline carboxypeptidase preparations obtained by the method of Anson (3). Some of the results of this investigation are reported in Table I. It will be noted that the amount of enzyme required for the hydrolysis of a substrate varies widely with the nature of the substrate.

The carbobenzoxyglycyl derivatives of several amino acids may be compared first. While carbobenzoxyglycylglycine is hydrolyzed rather slowly in the presence of 0.5 mg. of enzyme N per cc., carbobenzoxyglycyl-*l*-alanine requires only 0.08 mg. of enzyme N per cc. for a rather rapid hydrolysis. Finally, the carbobenzoxyglycine derivatives of *l*-phenylalanine and *l*-tyrosine are split in solutions containing only 0.3 γ of enzyme N per cc. The carbobenzoxyglycyl derivatives of *l*-phenylalanine and

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l-tyrosine are somewhat more sensitive toward carboxypeptidase than are the carbobenzoxy-*l*-glutamyl derivatives of these amino acids.

In order to reinvestigate the significance of the terminal carboxyl group, carbobenzoxy-*l*-glutamyl-*l*-phenylalanine and carbobenzoxy-*l*-glutamyl-*l*-phenylalanineamide were subjected to carboxypeptidase action. The relative amounts of enzyme employed for the experiments with the amide and the free acid were about 1000:1. Nevertheless, the amide was found to be resistant to enzymatic action, while the acid was hydrolyzed quite rapidly.

The typical substrates for carboxypeptidase do not contain any free amino group. It is therefore the more remarkable that α-hippuryl-ε-carbobenzoxy-*l*-lysine is not split by carboxypeptidase in contrast to the sensitivity of α-hippuryl-*l*-lysine. In this case, the peptide containing a free amino group is attacked by the enzyme. When the free amino group is masked, the peptide is rendered resistant toward the enzymatic action.

l-Tyrosyl-*l*-tyrosine and glycyl-*l*-tyrosine are other substrates for carboxypeptidase that contain free amino groups.

Benzoylglycylglycine is reported (1) to be resistant to the action of carboxypeptidase; however, it will be noted from Table I that when considerable amounts of the enzyme were employed, hydrolysis of this substrate could be effected. A still slower hydrolysis was found in the case of benzoyl-*l*-phenylalanine.

The fact that carbobenzoxyglycyl-*l*-glutamic acid is split rather slowly by carboxypeptidase seems also to be worthy of mention.

In the case of all the substrates hitherto subjected to the action of carboxypeptidase, the action of the enzyme has consisted in a splitting off of the amino acid residue bearing the terminal carboxyl group.

Chloroacetyl-*l*-tyrosine is generally regarded as the typical substrate for carboxypeptidase and is therefore frequently used to identify and to estimate this enzyme (10). The use of chloroacetyl-*l*-tyrosine for the estimation of carboxypeptidase is accompanied by two disadvantages. During the enzymatic hydrolysis free tyrosine crystallizes out, thus making the performance of a quantitative determination a difficult procedure. Of greater

TABLE I

Behavior of Synthetic Substrates toward Crystalline Carboxypeptidase

Substrate	Carboxypeptidase, 3 times crystallized	pH	Time	Hydrolysis		Isolation of products
				Titration	Van Slyke method	
	mg. N per cc.		*hrs.*	*per cent*	*per cent*	
Carbobenzoxyglycylglycine (4)*	0.510	7.5	24		30	
			48		50	
Carbobenzoxyglycyl-*l*-alanine (5)	0.083	7.3	1	66		
			2	81		
			10	102		
Carbobenzoxyglycyl-*l*-phenylalanine	0.00037	7.3	1	58	58	Carbobenzoxyglycine
			2	80	82	
			4	87		
			24	103	93	
Carbobenzoxyglycyl-*l*-tyrosine (6)	0.00037	7.7	1		37	*l*-Tyrosine
			2		†	
Carbobenzoxy-*l*-glutamyl-*l*-phenylalanine (7)	0.00037	7.4	1	13		
			2		19	
			6	31		
			7		37	
			24	61	61	
Carbobenzoxy-*l*-glutamyl-*l*-tyrosine (8)	0.00037	7.4	1		5	*l*-Tyrosine
			3		21	
			6		36	
			7		†	
Carbobenzoxy-*l*-glutamyl-*l*-phenylalanineamide*	0.520	7.9	24		0	
			48		5	
α-Hippuryl-ε-carbobenzoxy-*l*-lysine (9)*	0.520	7.4	24		8	
			48		8	
α-Hippuryl-*l*-lysine	0.208	7.4	1	23		Hippuric acid
			3	52		
			7	79		
			24	91		
l-Tyrosyl-*l*-tyrosine (8)	0.072	7.5	24		†	
	0.520	7.5	1		†	*l*-Tyrosine‡
Glycyl-*l*-tyrosine	0.645	7.5	4		11	"
			24		†	
Benzoylglycylglycine	0.520	7.2	5		64	Hippuric acid
			24		80	
Benzoyl-*l*-phenylalanine*	0.520	7.2	24		20	
			72		34	
Carbobenzoxyglycyl-*l*-glutamic acid*	0.510	7.4	24		12	
			72		38	
Chloroacetyl-*l*-tyrosine	0.00037	7.5	24		1	

* Temperature 25°.

† Tyrosine crystallization.

‡ NH_3-N, found 7.6 per cent.

significance is the fact that the other hydrolytic product, the chloroacetic acid, has an inhibiting effect upon the enzyme (see Table II). Consequently, the splitting of chloroacetyl-*l*-tyrosine by carboxypeptidase does not follow the course of a first order reaction. On the other hand, the hydrolysis of carbobenzoxy-glycyl-*l*-phenylalanine was found to accord rather closely with

TABLE II

Inhibition of Crystallized Carboxypeptidase by Chloroacetic Acid and Formaldehyde

Substrate	Carboxy-peptidase, 3 times crystallized	pH	Temperature	Time	Hydrolysis		
					No addition	Chloro-acetic acid*	Form-alde-hyde†
	mg. N per cc.		°*C.*	*hrs.*	*per cent*	*per cent*	*per cent*
Chloroacetyl-*l*-phenyl-alanine (11)	0.0039	7.2	40	3			2
				24			5
	0.0039	7.5	25	1	25	10	13
				2	36	18	15
				3	47	24	17
Carbobenzoxyglycyl-*l*-alanine	0.0720	7.3	40	3	73		2
				24	100		0
Carbobenzoxyglycyl-*l*-phenylalanine	0.00037	7.4	40	3	83		0
				24	94		4
	0.00024‡	7.6	25	1	16	9	4
				2	32	17	3
				3	45	26	3

* 0.05 mM sodium chloroacetate per cc. was added to the substrate solution before the enzyme.

† 0.2 cc. of 40 per cent formaldehyde (Merck) per cc. was added to the substrate solution before the enzyme.

‡ This enzyme solution was kept in the ice box for several weeks previous to its use and thus had lost some of its activity.

the kinetics of a first order reaction, as illustrated in Table III. The rate of reaction is approximately proportional to the enzyme concentration.

In view of the foregoing, carbobenzoxyglycyl-*l*-phenylalanine represents a very suitable substrate for determinations of carboxy-peptidase activity. 1 carboxypeptidase unit may be defined as that quantity of enzyme which, when dissolved in 1 ml. of a solution containing 0.05 mM of carbobenzoxyglycyl-*l*-phenylalanine,

under standard conditions[1] causes a hydrolysis with a reaction constant of $K = 0.0020$, where a is the initial concentration of substrate, $a - x$ is the concentration of substrate at time t (in minutes), and $K = 1/t \log a/(a - x)$.

The values reported in Table III demonstrate that our four times crystallized enzyme preparation had an activity corresponding to 6173 carboxypeptidase units per mg. of N. 1 unit was contained in about 1.1 γ of our crystalline carboxypeptidase.

TABLE III

Hydrolysis of Carbobenzoxyglycyl-l-Phenylalanine by Crystalline Carboxypeptidase at 25°

Carboxypeptidase, 4 times crystallized; pH 7.5	Time	Hydrolysis*	$K = \frac{1}{t} \log \frac{a}{a-x}$
mg. N per cc.	*min.*	*per cent*	
0.000097	60	15	0.0012
	120	28	0.0012
	180	40	0.0012
0.000194	30	15	0.0024
	60	29	0.0025
	90	41	0.0025
	120	49	0.0024
	180	63	0.0024
	240	77	0.0027
0.000388	30	32	0.0056
	60	53	0.0055
	90	68	0.0055
	120	77	0.0053

* Estimated in 1 cc. samples by NH_2-N determination.

In studies of the stability of carboxypeptidase under various conditions, the enzymatic activity is usually tested at the beginning and at the end of each treatment. In tests of this kind it is essential to begin each experiment with an enzyme concentration so low that a decrease of the enzyme concentration during the experiment must result in a decrease of the speed of the enzymatic hydrolysis. The initial enzyme concentration must be a different one for each synthetic substrate, because of the

[1] See "Experimental."

fact that the various substrates differ widely in their sensitivity toward carboxypeptidase. Failure to take this important factor into account may lead to erroneous conclusions. Abderhalden and Abderhalden (2) have found that a solution containing 1 per

TABLE IV

Partial Inactivation of Crystalline Carboxypeptidase

Two solutions, A and B, of twice crystallized carboxypeptidase were kept at 40° and tested for activity by means of the hydrolysis of synthetic substrates at regular intervals in the usual manner.

Experiment No.	Substrate	Initial enzyme concentration	Time of inactivation at 40°	pH	Hydrolysis	
					During 1 hr. at 40°	During 2 hrs. at 25°
Solution A, 1.04 mg. N per cc. in M/3 disodium phosphate; pH 8.3						
		mg. N per cc.	*hrs.*		*per cent*	*per cent*
1	Carbobenzoxy-glycyl-*l*-alanine	0 083	0	7.3	67	
			1		46	
			2		30	
2	Chloroacetyl-*l*-tryptophane	0.016	0	7.3	65	
			1		38	
			2		24	
3	"	0.083	0	7.4	94	
			2		88	
Solution B,* 0.0243 mg. N per cc. in 5 per cent sodium chloride; pH 7.5						
4	Chloroacetyl-*l*-phenylalanine	0.0049	0	7.6		45
			1			29
			2			19
5	Carbobenzoxy-glycyl-*l*-phenylalanine	0.00024	0	7.6		40
			1			24
			2			14
6	"	0.0049	2	7.5		98

* This enzyme solution was kept in the ice box for several weeks before its use and thus had lost some activity.

cent of "carboxypeptidase," when kept at 56°, became inactive toward β-naphthalenesulfonylglycyl-*l*-tyrosine much sooner than toward chloroacetyl-*dl*-leucine. This finding was interpreted as indicating that the two substrates are split by two different enzymes.

It will be apparent from Experiments 1 and 2 of Table IV that, when the initial enzyme concentrations are adapted to the sensitivity of the substrates, no indication of an enzymatic inhomogeneity can be observed. It will be noticed that at 40° the activities toward chloroacetyl-*l*-tryptophane and carbobenzoxyglycyl-*l*-alanine are destroyed at about identical rates.[3] A comparison of Experiments 1 and 3, however, shows that, when identical initial enzyme concentrations are employed for both substrates, the activity toward carbobenzoxyglycyl-*l*-alanine disappears much more rapidly. In Experiment 3 the initial enzyme concentration represents a huge excess.

In Experiments 4 to 6 the inactivation was effected under slightly different conditions. Chloroacetyl-*l*-phenylalanine and carbobenzoxyglycyl-*l*-phenylalanine were employed as substrates. When the appropriate initial enzyme concentrations were employed, the rate of enzyme destruction was approximately the same for the two substrates; if identical enzyme concentrations were used for both substrates, as in Experiments 4 and 6, the activity toward chloroacetyl-*l*-phenylalanine appeared to be destroyed more rapidly.

In the last analysis, all comparative enzyme experiments performed with the aid of simple substrates consist in a comparison of reaction velocities. Therefore, the initial enzyme concentration must be sufficiently low so that the observed hydrolysis can be taken as a true expression of the quantity of active enzyme. Experiments 3 and 6 of Table IV and the previously discussed experiments of Abderhalden do not satisfy this condition, and therefore cannot be interpreted as indicating an enzymatic inhomogeneity of crystalline carboxypeptidase.

With the use of various synthetic substrates, the action of formaldehyde upon carboxypeptidase has been studied. The experiments reported in Table II demonstrate that formaldehyde inactivates carboxypeptidase at 40° and also at 25°.

The authors wish to express their thanks to Mr. Stephen M. Nagy who performed the analyses reported in this paper.

[3] At 40° the destruction of carboxypeptidase proceeds more slowly than at 56°.

EXPERIMENTAL

Carbobenzoxyglycyl-l-Phenylalanine—To an ice-cold ethereal solution of *l*-phenylalanine ethyl ester (prepared from 3.8 gm. of the hydrochloride) there were added, in two portions, 3.8 gm. of carbobenzoxyglycyl chloride dissolved in ether, the second portion being followed by 50 cc. of a saturated aqueous solution of potassium bicarbonate. The mixture was shaken for 30 minutes and kept at room temperature for 1 hour. 2 cc. of pyridine were then added, and the reaction mixture was worked up in the usual manner. On saponification, the corresponding oily ester yielded 4 gm. of crystals which were purified by crystallization from a mixture of ethyl acetate and ether. M.p., 125–126°.

$C_{19}H_{20}O_5N_2$.	Calculated.	C 64.0, H 5.6, N 7.8
356.4	Found.	" 63.7, " 5.6, " 7.7

$[\alpha]_D^{24} = +38.5°$ (5% in ethyl alcohol)

178 mg. of this compound were hydrolyzed by carboxypeptidase. The hydrolysate was acidified to Congo red, filtered, and concentrated to a small volume *in vacuo*. The concentrate was then extracted several times with ethyl acetate. The combined ethyl acetate extracts were washed with water, dried over Na_2SO_4, and concentrated, yielding 100 mg. of crystals (96 per cent of the theory) which were purified by crystallization from a mixture of ether and petroleum ether. M.p., 120–121°. The mixed melting point with carbobenzoxyglycine was 120–121°.

$C_{10}H_{11}O_4N$ (209.2). Calculated, N 6.7; found, N 6.7

Estimation of Carboxypeptidase—To 445 mg. of carbobenzoxyglycyl-*l*-phenylalanine are added 2.5 cc. of M/3 phosphate buffer of pH 7.6, 1.25 cc. of N sodium hydroxide, and enough water to make the total volume 10 cc. 2 cc. of the above substrate solution are mixed with the carboxypeptidase solution to be tested, and the volume is made up to 5 cc. with water. The reaction mixture is kept at 25° and 1 cc. aliquots are withdrawn for amino nitrogen estimation at the beginning and at 1 and 2 hour intervals. 100 per cent hydrolysis of the substrate corresponds to an increase of 0.7 mg. of NH_2-N. In the performance of such an estimation, the enzyme concentration in the test solution should

be so adjusted that, under the above conditions, the extent of hydrolysis in 1 hour is 10 to 30 per cent.

Carbobenzoxyglycyl-l-Glutamic Acid—An ethyl acetate solution of *l*-glutamic acid diethyl ester (prepared from 2.4 gm. of the hydrochloride) was coupled with 1.1 gm. of carbobenzoxyglycyl chloride, as described for carbobenzoxyglycyl-*l*-phenylalanine. The corresponding ester (2.9 gm.) was dissolved in 20 cc. of N NaOH by the addition of a few cc. of methanol, and allowed to stand for 2 hours. The solution was then acidified to Congo red with N HCl and concentrated to a small volume *in vacuo*. The crystals which had separated out were filtered off and purified by crystallization from hot water. M.p., 160–162°; yield, 1.2 gm.

$C_{15}H_{18}O_7N_2$.	Calculated.	C 53.3, H 5.4, N 8.3
338.3	Found.	" 53.3, " 5.4, " 8.1

$[\alpha]_D^{24} = +4.0°$ (5% in ethyl alcohol)

Carbobenzoxy-l-Glutamyl-l-Phenylalanineamide—1 gm. of carbobenzoxy-*l*-glutamyl-*l*-phenylalanine ethyl ester (7) was dissolved in 100 cc. of methanol which had previously been saturated with dry ammonia. The solution was kept 3 days at room temperature and was then concentrated *in vacuo*. The residue was taken up in water and acidified to Congo red with N HCl. The crystals which separated out were filtered off, dried, and recrystallized from methanol. Needles which melted at about 185–187° were obtained. Yield, 500 mg.

$C_{22}H_{25}O_6N_3$.	Calculated.	C 61.8, H 5.9, N 9.8
427.4	Found.	" 61.6, " 5.8, " 9.9

α-Hippuryl-l-Lysine—2 gm. of α-hippuryl-ε-carbobenzoxy-*l*-lysine (9) were dissolved in 20 cc. of methanol and hydrogenated in the presence of a palladium catalyst. The catalyst was then filtered off and the filtrate concentrated *in vacuo*, yielding a syrup which soon crystallized. The material, which was purified by crystallization from a mixture of water and dioxane, consisted of needles that melted at 236–238°. Yield, 1 gm.

$C_{15}H_{21}O_4N_3$.	Calculated.	C 58.6, H 6.9, N 13.6
307.4	Found.	" 58.6, " 6.8, " 13.5 (Dumas)

$[\alpha]_D^{24} = -5.2°$ (2.5% in H_2O)

192 mg. of this compound were hydrolyzed by carboxypeptidase. The hydrolysate was acidified, with Congo red as indicator, and evaporated *in vacuo*. The crystals thus obtained were recrystallized from hot water and melted at 188–190°. Yield, 93 mg. (83 per cent of the theory). The mixed melting point with hippuric acid was 188–190°.

$C_9H_9O_3N$ (179.2). Calculated, N 7.8; found, N 7.7

Benzoylglycylglycine—This compound was prepared as described by Fischer (12).

118 mg. of benzoylglycylglycine were hydrolyzed by carboxypeptidase. 76 mg. (85 per cent of the theory) of hippuric acid were isolated from the hydrolysate in the manner previously described under the splitting of α-hippuryl-*l*-lysine. The product melted at 188–190°. The mixed melting point with hippuric acid was 188–190°.

$C_9H_9O_3N$ (179.2). Calculated, N 7.8; found, N 7.7

Enzymatic Studies

The crystalline carboxypeptidase was prepared and recrystallized according to the directions of Anson (3). The substrates containing a carboxyl group were used in the form of their sodium salts. The concentration of the substrates was 0.05 mM per cc. in all cases. The solutions were buffered by M/30 phosphate buffers. All the pH values were measured by the glass electrode. Except where otherwise stated, the temperature was 40°. The extent of hydrolysis was followed either by determining the amino nitrogen liberated in the Van Slyke microvolumetric apparatus or by measuring the liberated carboxyl groups according to the method of Grassmann and Heyde (13). Enzyme blanks and tests for the lability of the substrate in the absence of carboxypeptidase were performed.

BIBLIOGRAPHY

1. Oppenheimer, C., Die Fermente und ihre Wirkungen, The Hague, 5th edition, suppl., 764 (1936).
2. Abderhalden, E., and Abderhalden, R., *Fermentforschung*, **16**, 48 (1938).
3. Anson, M. L., *J. Gen. Physiol.*, **20**, 663 (1937).
4. Bergmann, M., and Zervas, L., *Ber. chem. Ges.*, **65**, 1200 (1932).
5. Bergmann, M., and Fruton, J. S., *J. Biol. Chem.*, **117**, 201 (1937).

6. Bergmann, M., and Fruton, J. S., *J. Biol. Chem.*, **118**, 413 (1937).
7. Fruton, J. S., and Bergmann, M., *J. Biol. Chem.*, **127**, 635 (1939).
8. Bergmann, M., Zervas, L., Salzmann, L., and Schleich, H., *Z. physiol. Chem.*, **224**, 17 (1934).
9. Hofmann, K., and Bergmann, M., *J. Biol. Chem.*, **130**, 85 (1939).
10. Anson, M. L., *J. Gen. Physiol.*, **20**, 781 (1937).
11. Fischer, E., and Schoeller, W., *Ann. Chem.*, **357**, 20 (1907).
12. Fischer, E., *Ber. chem. Ges.*, **38**, 614 (1905).
13. Grassmann, W., and Heyde, W., *Z. physiol. Chem.*, **183**, 32 (1929).

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DETERMINATION OF PROLINE IN MIXTURES CONTAINING *l*- AND *dl*-PROLINE

The Proline Content of Gelatin

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In a recent communication (1) there was described a method for the determination of amino acids, based on the law of the constancy of the solubility product. With the aid of this method, and with ammonium rhodanilate as reagent, an *l*-proline content of 17.5 per cent was found for various gelatins and for tendon collagen. It was doubtful whether this figure represented the total proline content of the proteins investigated; for, if partial racemization of proline occurred during the hydrolysis of the proteins with boiling HCl, the presence of the *d* isomer might not have been detected. In order to clarify this point, studies were undertaken of the behavior of mixtures of *l*- and *d*-proline when precipitated with ammonium rhodanilate under the conditions employed in the estimation of proline by the solubility method.

It was found that, from a solution containing equimolecular amounts of *l*- and *d*-proline, there was precipitated a *dl*-rhodanilate[1] which was found to be considerably less soluble, in the methanol-water solvent employed, than is *l*-proline rhodanilate. The solubility product constant of *l*-proline rhodanilate is 9.2×10^{-6}, whereas the constant for *dl*-proline rhodanilate is 3.1×10^{-6}. It follows that the latter precipitate is a racemic compound, and not an inactive mixture of *l*- and *d*-proline rhodanilates.

When mixtures of *l*- and *dl*-proline were dissolved in aqueous HCl and a methanol solution of ammonium rhodanilate was added, the precipitates obtained consisted of mixtures of *l*-proline

[1] All the experiments with proline rhodanilate discussed in this paper were performed at 0°.

rhodanilate and its racemate. A comparison of Columns 3 and 4 of Table I reveals that the relative proportion of *l*- and *dl*-proline found, as rhodanilates, in the precipitate is in each case about the same as in the corresponding original mixture. Even when *dl*-proline comprises 90 per cent of the original mixture, no marked fractionation occurs in the course of the precipitation of the rhodanilates. It appears, therefore, that the law of the constancy of the solubility product is not the sole factor governing the

TABLE I

*Precipitation of Mixtures of l- and dl-Proline with Ammonium Rhodanilate**

Proline employed (1)	Proline rhodanilate pptd.† (2)	*l*-Proline rhodanilate in ppt. (3)	*l*-Proline in original mixture‡ (4)
mole	*mole*	*per cent*	*per cent*
0.00386 *l*- + 0.00021 *dl*-	0.00337	97.8	95.8
0.00386 *l*- + 0.00042 *dl*-	0.00347	93.9	90.2
0.00386 *l*- + 0.00063 *dl*-	0.00359	88.7	86.2
0.00313 *l*- + 0.00090 *dl*-	0.00341	74.4	77.7
0.00044 *l*- + 0.00361 *dl*-	0.00363	10.6	10.9

* For the experimental procedure, volume, and composition of solvent, etc., see the text.

† The amount of ammonium rhodanilate added was, in all cases, about 120 per cent of the theoretical quantity necessary to precipitate all the proline present.

‡ Calculated from Column 1.

equilibrium in this case. The data suggest that *dl*- and *l*-proline rhodanilates form solid solutions in all proportions.

Further indication of the presence of solid solutions is afforded by the data in Table II. Four mixtures of *l*- and *dl*-proline rhodanilate, of the composition indicated in Column 1, were dissolved in methanol and precipitated by the addition of aqueous HCl. The solid phase obtained was analyzed polarimetrically for its content of *l*- and *dl*-proline rhodanilate as described in a previous communication (1). The resulting data on the composition of the solid phase at equilibrium are given in Column 2,

and its solubility in Column 3. The solubilities of pure *dl*- and *l*-proline rhodanilates were measured under the same conditions, and are represented by the first and last figures in Column 3. The values in Column 4 are the solubilities calculated for solid solutions of the *l* and *dl* salts. In this calculation, the solubility, S, of a solid solution in the liquid phase is considered to be $S = M_l S_l + M_{dl} S_{dl}$, where M_l and M_{dl} represent the mole fractions of the *l* and *dl* components in the solid phase at equilibrium, and S_l and S_{dl} are the solubilities of the pure *l* and *dl* components

TABLE II

*Solubility of Solid Solutions of l- and dl-Proline Rhodanilates**

Composition of mixture of rhodanilates employed† (1)	Composition of solid phase at equilibrium (2)	Solubility of solid phase at equilibrium	
		Found (3)	Calculated‡ (4)
per cent	*per cent*	*mg. per 330 cc.*	*mg. per 330 cc.*
100 *dl*-	100 *dl*-	297	
80 " + 20 *l*-	79.5 *dl*- + 20.5 *l*-	329	344
60 " + 40 "	65.4 " + 34.6 "	373	377
40 " + 60 "	45.2 " + 54.8 "	428	424
20 " + 80 "	20.6 " + 79.4 "	489	481
100 *l*	100 *l*-	529	

* For the experimental procedure, volume, and composition of solvent, etc., see the text.

† The amount of original mixture employed was, in all cases, about 2.0 gm.

‡ In calculating the solubilities in Column 4, the molecular weight of *dl*-proline rhodanilate is assumed to be the same as that of *l*-proline rhodanilate.

in the water-methanol solvent. Meyer (2) has found that this simple equation, which follows from Raoult's law, best fits the solubility data yielded by solid solutions of two binary electrolytes having a common ion. From the data obtained here for the proline rhodanilates, it is observed that the solubilities calculated in this manner are in close agreement with the experimental values.

A consideration of the foregoing experiments with known mixtures indicates that it should be possible readily to detect the presence of *d*-proline in a protein hydrolysate. This premise was checked on a gelatin hydrolysate in the following manner.

To a hydrolysate of 2.59 gm. of water-free gelatin (corresponding to about 450 mg. of *l*-proline) there were added 34 mg. of *dl*-proline (corresponding to about 7.1 per cent of the total proline content). The proline rhodanilate precipitate obtained in the usual manner was analyzed polarimetrically and found to contain 91.8 per cent of *l*-proline rhodanilate and 8.2 per cent of *dl*-proline rhodanilate. The proline rhodanilate obtained from another sample of the same hydrolysate, to which no *dl*-proline had been added, was found to contain 99.1 per cent of pure *l*-proline rhodanilate.

From the above information it may be concluded that in the hydrolysates of various gelatins and of collagen that have been analyzed recently (1), the *d* isomer content could not have been more than about 1.5 per cent of the total proline. All the precipitates of proline rhodanilate obtained in the course of these earlier analytical procedures were examined polarimetrically, and in all cases a rotation corresponding to 97 to 100 per cent of *l*-proline rhodanilate was observed. An appreciable amount of the *d* or the *dl* form in the hydrolysate should have manifested itself in a lower rotation of the rhodanilates.

Although it has been shown that the hydrolysates investigated earlier contained only a negligible amount of *d*- or *dl*-proline, it will be demonstrated later that during prolonged hydrolysis of gelatin with boiling HCl appreciable racemization of proline occurs. It is desirable to ascertain, therefore, whether it is possible, by the solubility method, to determine the total proline content of a mixture containing *l*- and *dl*-proline. To achieve such a determination, it is a necessary condition that the solubility product of the proline rhodanilate be a constant, despite the stereochemical inhomogeneity of the proline.

The results of two determinations on known mixtures, the one containing 22.3 per cent of *dl*-proline and 77.7 per cent of *l*-proline, the other containing 89.1 per cent of *dl*-proline and 10.9 per cent of *l*-proline, are recorded in Table III. A consideration of the data in Table III indicates that the above condition is fulfilled, and that a satisfactory determination of the total proline content of such mixtures is possible. The proline content found (Column 2) was calculated simply from the weights of the proline rhodanilate precipitates obtained in the course of the determination, without regard to their stereochemical character.

When determinations of the total proline content of proteins are performed, it is important to ascertain the optimum conditions of hydrolysis and to know whether proline is destroyed or racemized in the course of prolonged hydrolysis. In order to check this point the proline content of a gelatin hydrolysate was determined after 3 hours of hydrolysis with boiling concentrated HCl.[3] The total proline content was found to be 17.0 and 17.3 per cent. The proline rhodanilate precipitates obtained in these determinations exhibited the rotation of the pure *l* form. These results are in close agreement with the value, 17.5 per cent, previously obtained after 8 hours of hydrolysis.

TABLE III

Determination of Total Proline Content of Solutions of l- and dl-Proline

Proline content of solutions employed (1)	Total proline content found (2)	Average recovery (3)	Solubility product* constant (4)	*l*-Proline rhodanilate content of ppts. (5)
mole	*mole*	*per cent*	$\times 10^{-6}$	*per cent*
0.00313 *l*- +	0.00403		8.5	73.5
0.00090 *dl*-	0.00391	97.5	8.3	73.9
	0.00382		9.4	74.4
0.00361 *dl*- +	0.00424	104	3.8	7.9
0.00044 *l*-			3.2	10.6

* The solubility products are, in each case, the product of the concentrations of the proline and the rhodanilic acid remaining in the solutions. The concentration of the proline is considered to be the sum of the concentrations of the stereoisomers. *Cf.* also Bergmann and Stein ((1) p. 219, foot-note 1).

On the other hand, when gelatin was hydrolyzed under similar conditions for 48 hours and proline determined as before, each of the proline rhodanilate precipitates obtained gave the theoretical C and H values for proline rhodanilate and contained 86.6 per cent of *l*-proline rhodanilate and 13.4 per cent of *dl*-proline rhodanilate. The total proline content of the hydrolysate, however, was found to be 17.1 per cent, in good agreement with the values obtained after 3 and 8 hours hydrolysis. Apparently a portion of the

[3] The ratio NH_3-N to total N was 0.63. After the hydrolysate was cleared by the CuS procedure (1), the ratio NH_3-N to total N had risen to 0.66.

proline was racemized during 48 hours hydrolysis, but no appreciable amount was destroyed.

Experiments of this type naturally do not indicate whether some proline is destroyed in the peptide stage during the first 3 hours of the hydrolysis. In the absence of evidence for destruction of proline in the peptide stage, the value 17.5 per cent (±0.5 per cent) may be regarded as representing the proline content of gelatin and tendon collagen.

EXPERIMENTAL

Precipitation of Mixtures of l- and dl-Proline with Ammonium Rhodanilate—In order to demonstrate the way in which the data in Table I were obtained, the first experiment in the table may be described. 555 mg. of *l*-proline and 30 mg. of *dl*-proline were dissolved in water, 35 cc. of N HCl were added, and the volume was made up to 250 cc. The solution was cooled, and a 200 cc. aliquot (containing 0.00386 mole of *l*-proline and 0.00021 mole of *dl*-proline) was added to 2.505 gm. of ammonium rhodanilate dissolved in 130 cc. of cold methanol. The solution was agitated at 0°, in a tightly stoppered flask, for 48 hours. The precipitate was collected at 0° on weighed crucibles with sintered glass filter plates, and dried over $CaCl_2$ and KOH in a desiccator to constant weight. The precipitate was analyzed polarimetrically in the manner already described.[3]

Solubility of Solid Solutions of l- and dl-Proline Rhodanilates—2.000 gm. samples of the mixtures of the composition indicated in Column 1, Table II, were dissolved in 130 cc. of cold methanol, and 200 cc. of ice-cold water containing 25 cc. of N HCl were added to each. The mixtures were agitated at 0° for 48 hours, and the precipitates were filtered, dried, weighed, and analyzed polarimetrically. The results of the polarimetric analysis are reported in Column 2, Table II. The solubilities, reported in Column 3, Table II, were determined as the difference in weight between the mixtures employed and the precipitates recovered.

[3] In our earlier communication (1), the equation for calculating the *l*-proline content of a sample of proline rhodanilate subjected to polarimetric analysis was given erroneously. The equation should read $p = (\alpha \times S \times 57)/(-1.52° \times 3.19)$ (p represents proline in mg., α observed rotation, and S total weight of solvents in gm.).

The precipitate containing 79.5 per cent of *dl*-proline and 20.5 per cent of *l*-proline and the one containing 20.6 per cent of *dl*-proline and 79.4 per cent of *l*-proline were analyzed for their carbon and hydrogen content.

$(C_{16}H_{14}N_6S_4Cr)\cdot(C_5H_{10}O_2N)\cdot H_2O$.	Calculated.	C 41.7, H 4.3
604.6	Found.	" 41.8, " 4.3
	"	" 41.7, " 4.4

Quantitative Determination of Total Proline Content of Mixtures of l- and dl-Proline—515 mg. of *dl*-proline, 1.800 gm. of *l*-proline, and 140 cc. of N HCl were made up to 1 liter with water. The solution was cooled, and three 200 cc. aliquots, each containing 0.00403 mole of total proline, were added to 1.800, 2.202, and 2.603 gm. samples of ammonium rhodanilate each dissolved in 130 cc. of cold methanol. After 48 hours agitation at 0°, the precipitates were collected, dried, weighed, and analyzed polarimetrically. The precipitates weighed 1.675, 1.929, and 2.062 gm. and contained 73.5, 73.9, and 74.4 per cent of *l*-proline rhodanilate and 26.5, 26.1, and 25.6 per cent of *dl*-proline rhodanilate respectively. The three calculated values for the total proline content of the aliquots taken were 0.00403, 0.00391, and 0.00382 mole; average 0.00392 mole, corresponding to 97.5 per cent of the theory.

The analysis of the other mixture mentioned in the text was performed in the same manner.

BIBLIOGRAPHY

1. Bergmann, M., and Stein, W. H., *J. Biol. Chem.*, **128**, 217 (1939).
2. Meyer, G., *Rec. trav. chim. Pays-Bas*, **42**, 301 (1923).

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THE REACTION OF THE ESTERS OF *dl*-LEUCINE AND OF *l*-LEUCINE ON THE RANEY CATALYST

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Catalytic reduction of esters of α-amino acids to corresponding alcamines or N-alkylated alcamines over copper chromite catalyst was accomplished earlier in this laboratory. It was realized, however, that the nickel catalyst has a wider temperature range of activity and that therefore by changing the conditions of the reaction, the process might be controlled so that, on one hand, the desired product would be obtained and, on the other hand, the mechanism of the more complex reactions would be brought to light. Indeed, it was hoped that, by means of reduction over Raney's catalyst, conditions might be found to obtain from the esters of optically active amino acids optically active alcamines. These in their turn might serve, on one hand, for the study of the relationship of optical activity to physiological action (since derivatives of alcamines are physiologically active) and, on the other hand, as an intermediary for the assignment of the configurations of α-amino acids whose configurations have not yet been established by direct chemical methods. The expectations were realized in many ways, although further refinement in procedure is possible.

The present communication deals with the results of experiments on the ethyl ester of *dl*-leucine and of *l*-leucine. The earlier experiments were performed on the *dl* form of the acid for obvious reasons.

The conditions varied with respect to temperature, duration, and proportion of catalyst.

At $t = 135°$ and an initial pressure of 2200 pounds per sq. inch the only product isolated was leucinol.

At t = 185° and at 200° and the same initial pressure, the following products were formed: N-dimethyl-2-amino-4-methylpentane, N-dimethylleucinol, 2,5-diisobutylpiperazine, and N,N′-dimethyl-2,5-diisobutylpiperazine. In none of the experiments were all products formed but the number of them and their pro-

TABLE I

Reduction of Leucine Ethyl Ester and Its Derivatives and of Glycylglycine Anhydride in Hydrogen over Raney's Catalyst at Varying Temperatures and at 2200 Pounds Initial Pressure

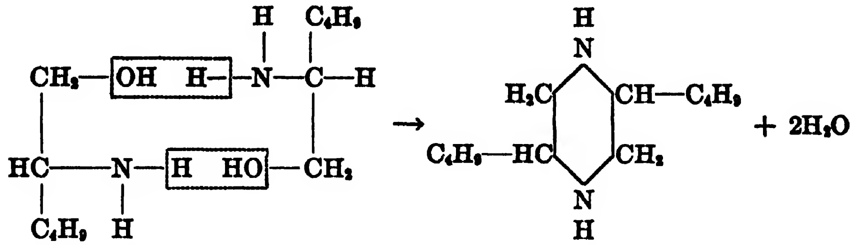
Experiment No.	Starting substance	Weight	Duration	Temperature	Yield: N-Dimethyl-2-amino-4-methylpentane	Yield: 2,5-Diisobutylpiperazine hydrochloride	Yield: Leucinol hydrochloride	Yield: N-Dimethylleucinol	Yield: N,N′-Dimethyl-2,5-diisobutylpiperazine
		gm.	*hrs.*	*°C.*	*gm.*	*gm.*	*gm.*	*gm.*	*gm.*
1	Leucine ethyl ester	2	9	70			0.6*		
2	" " "	9	22	135			3.8		
3	" " "	10	4	185		1.2		6	1.3
4	" " "	10	12	185		0.9		6	2.5
5	" " "	10	24	185	2			4.5	2.5
6	" " "	5	16	200	0.5			1.3	2.2
7	N-Dimethylleucinol	4.5	10	185	2.6				
8	Leucinol	3	12	185	1.9				
9	Leucylleucine anhydride	3	16	200					2.7
10	Glycylglycine anhydride	3	16	200					2.1†

* Leucinol.
† N,N′-Dimethylpiperazine.

portions varied with change in duration of the reaction and in temperature. The proportions are given in Table I.

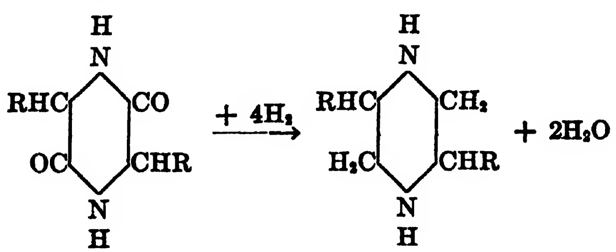
As the work progressed, the importance of the proportion of catalyst used became evident and we were encouraged to attempt the reduction of the ethyl ester of *l*-leucine at t = 70° with a large excess of the catalyst in the hope of obtaining the optically active *l*-leucinol. This hope likewise was realized.

The mechanism of the formation of the piperazines attracted special attention. It was logical to expect that leucinol and dimethylleucinol should be the primary products of the reaction, on the basis of the experience of Paden and Adkins[1] and of Hill



and Adkins.[2] Indeed, Bain and Pollard[3] observed the condensation of amino alcohols into piperazines on heating over Adkins' catalyst at 235°. However, by means of Raney's catalyst under the conditions of temperature and pressure employed by us, both leucinol and dimethylleucinol failed to yield detectable quantities of piperazines, only 2-aminohexane and N-dimethyl-2-aminohexane respectively having been isolated as products of the reaction. Hence the mechanism of the formation of piperazines required further explanation.

It was then realized that part of the leucine ester on heating in methanol solution might condense into a ketopiperazine which then might be reduced to the corresponding piperazine, which would or would not be methylated, depending on the conditions of the experiment. This expectation was fully realized, for it

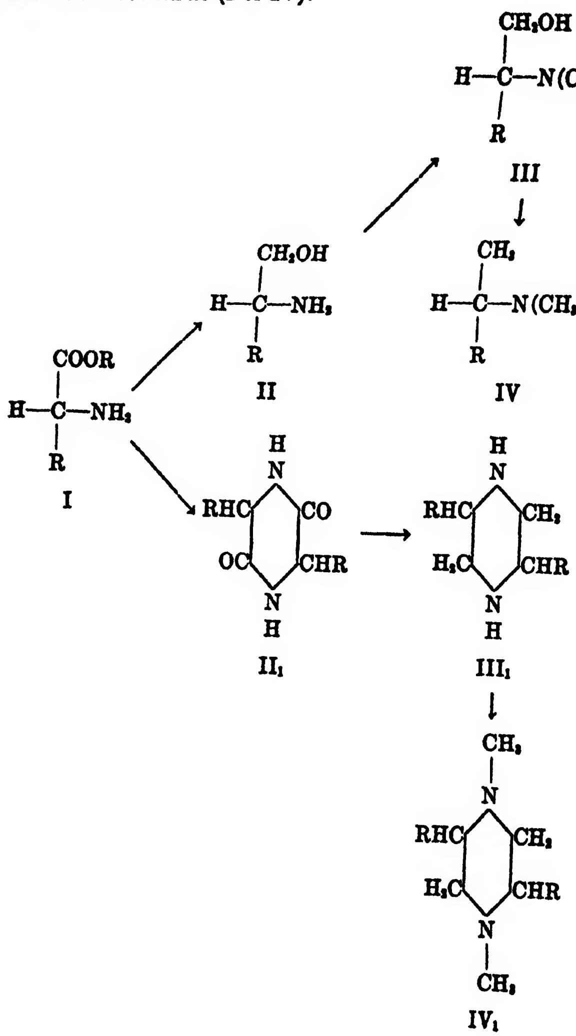


was found by experiment that, on one hand, the ester heated in a sealed tube in methanol solution in the presence or in the

[1] Paden, J. H., and Adkins, H., *J. Am. Chem. Soc.*, **58**, 2487 (1930).
[2] Hill, R. M., and Adkins, H., *J. Am. Chem. Soc.*, **60**, 1033 (1938).
[3] Bain, J. P., and Pollard, C. B., *J. Am. Chem. Soc.*, **61**, 532 (1939).

absence of hydrogen condensed into the corresponding ketopiperazine and, on the other, that the latter was reduced to the corresponding piperazine when heated over Raney's catalyst at 185° in an atmosphere of hydrogen.

Thus at 185° and at an initial pressure of 2200 pounds of hydrogen per sq. inch two primary products are formed simultaneously, each undergoing further reaction. The course of the reaction is presented in formulae (I to IV).



SUMMARY

1. Conditions are given for the catalytic reduction over Raney's catalyst of *dl*-leucine ethyl ester to *dl*-leucinol and for the conversion of *l*-leucine ethyl ester to *l*-leucinol.

2. Conditions are given for separation of the products formed from leucine ethyl ester over Raney's catalyst at the temperature of 185°. The products are N-dimethyl-2-amino-4-methylpentane, N-dimethylleucinol, 2,5-diisobutylpiperazine, and N,N'-dimethyl-2,5-diisobutylpiperazine.

3. The mechanism of formation of piperazines from esters of amino acids over Raney's catalyst is discussed.

4. A convenient procedure for the preparation of ketopiperazines is given.

EXPERIMENTAL

Action of Raney's Catalyst on l-Leucine Ethyl Ester in Presence of Hydrogen at 70°—5 gm. of *l*-leucine were converted into its ethyl ester in the usual manner. Yield 3.8 gm.

4.680 mg. substance: 10.310 mg. CO_2 and 4.140 mg. H_2O
7.081 " " : 0.539 cc. N_2 at 27°, p 761 mm.

$C_8H_{17}O_2N$.	Calculated.	C 60 37, H 10.69, N 8.80
159.1	Found.	" 60.07, " 10.62, " 8.67

$$[\alpha]_D^{25} = \frac{+10.8°}{1 \times 0.92} = +11.7° \text{ (homogeneous)}$$

4 gm. of Raney's catalyst were added to a solution of 1 gm. of *l*-leucine ethyl ester, $[\alpha]_D^{25} = +11.7°$, in 40 cc. of absolute methanol and reduced with hydrogen at a pressure of 2200 pounds per sq. inch at 70° for 9 hours. The product was isolated as usual. B.p. 130°, $p = 18$ mm.

The substance had a composition agreeing with that calculated for *l-leucinol.*

3.485 mg. substance: 7.800 mg. CO_2 and 4.065 mg. H_2O
4.185 " " : 0.438 cc. N_2 at 28°, p 757 mm.

$C_6H_{15}ON$.	Calculated.	C 61.47, H 12.90, N 11.95
117.1	Found.	" 61.10, " 13.02, " 11.85

$$[\alpha]_D^{25} = \frac{+0.27° \times 100}{1 \times 7.1} = +3.8° \text{ (in methanol)}; \; n_D^{25} = 1.4476$$

l-Leucinol Picrate—0.2 gm. of distilled *l*-leucinol was dissolved in 1 cc. of ether and an ether solution of 0.2 gm. of dry picric acid was added. After standing for several hours the product had completely crystallized.

A constant melting point of 120–121° was reached after recrystallization from methanol.

The substance had a composition agreeing with that calculated for the *l-leucinol picrate.*

5.276 mg. substance: 8.085 mg. CO_2 and 2.525 mg. H_2O
4.875 " " : 0.691 cc. N_2 at 28°, *p* 761 mm.

$C_{12}H_{18}O_8N_4$.	Calculated.	C 41.61, H 5.26, N 16.18
346.1	Found.	" 41.78, " 5.36, " 16.10

$$[\alpha]_D^{25} = \frac{+0.27° \times 100}{1 \times 4.6} = +5.9° \text{ (in methanol)}$$

In another experiment, 2 gm. of leucine ethyl ester, $[\alpha]_D^{25} = +10.6°$ (homogeneous), were dissolved in 80 cc. of methanol and 5.5 cc. of centrifuged Raney's catalyst (8 gm.) were added. This was reduced at 2200 pounds pressure for 9 hours at 70°.

The product distilled from a bath at a temperature of 100–110° at a pressure of 12 mm. Weight 0.6 gm., $n_D^{25} = 1.4478$.

$$[\alpha]_D^{25} = \frac{+0.23° \times 100}{1 \times 12} = +1.9° \text{ (in methanol)}$$

4.425 mg. substance: 9.995 mg. CO_2 and 5.080 mg. H_2O

$C_6H_{15}ON$.	Calculated.	C 61.47, H 12.90
117.1	Found.	" 61.59, " 12.84

Action of Raney's Nickel Catalyst on dl-Leucine Methyl Ester in Methanol at 135°—9 gm. of *dl*-leucine methyl ester were dissolved in 100 cc. of methyl alcohol and reduced for 24 hours with Raney's catalyst in hydrogen at a temperature of 135° and an initial pressure of 2200 pounds per sq. inch. The catalyst was removed by filtration and the filtrate was acidified with hydrogen chloride. The acidified solution was concentrated to a semicrystalline mass and then dissolved in 150 cc. of warm acetone. After cooling, the crystalline product was filtered. Yield 3.8 gm.

Recrystallized from methanol, the substance had the correct melting point for *dl*-leucinol hydrochloride, 160–161°,[4] and the following composition.

[4] Christman, C. C., and Levene, P. A., *J. Biol. Chem.*, **124**, 453 (1938).

4.914 mg. substance: 8.505 mg. CO_2 and 4.495 mg. H_2O

$C_6H_{15}ON \cdot HCl$.	Calculated.	C 46.87, H 10.5
153.6	Found.	" 47.19, " 10.3

The acetone mother liquors were concentrated, yielding a mixture of unchanged leucine methyl ester and leucinol.

Action of Raney's Nickel Catalyst on Leucine Methyl Ester in Methanol in Presence of Hydrogen at 185°. I. 2,5-Diisobutylpiperazine Hydrochloride—10 gm. of the ester were dissolved in 100 cc. of methyl alcohol and reduced with hydrogen in the presence of Raney's catalyst at the temperature of 185° and initial pressure of 2200 pounds per sq. inch. The reaction product was worked up as described above. The semicrystalline mass of the hydrochloride was treated with 50 cc. of warm methyl alcohol which dissolved all the material in some experiments and left some insoluble material in other experiments. The filtrate after the removal of the insoluble substance was worked up as given in section (II). The insoluble substance was dissolved in 10 cc. of water, cooled to 0°, and treated with 10 cc. of a cold 25 per cent sodium hydroxide solution. The free base was extracted with ether and the ether extract was dried with potassium carbonate. After removal of the ether a crystalline substance was obtained which was purified by three recrystallizations from pentane. This substance melted at 79–80° and had a composition agreeing with that calculated for a dibutylpiperazine.

3.222 mg. substance: 8.590 mg. CO_2 and 3.790 mg. H_2O

4.110 " " : 0.50 cc. N_2 at 27°, p 763 mm.

$C_{12}H_{26}N_2$.	Calculated.	C 72.72, H 13.2, N 14.1
198.2	Found.	" 72.64, " 13.2, " 13.9

II. N-Dimethyl-2-Amino-4-Methylpentane—After removal of the dibutylpiperazine hydrochloride, the methyl alcohol solution was concentrated to dryness and dissolved in 50 cc. of cold water. This solution was treated with 40 cc. of a cold 25 per cent sodium hydroxide solution and the free base was extracted with ether. The ether extract was dried with potassium carbonate and the ether distilled off through a 10 inch Vigreux column, the temperature of the bath being kept below 65°.

The crude material was then fractionally distilled through a 6 inch Vigreux column at a pressure of 20 to 22 mm. The first fraction distilled at 50–60° and was redistilled under atmospheric

pressure, boiling at 110–115°. This substance had a composition agreeing with that calculated for an N-dimethylaminohexane.

4.823 mg. substance: 13.109 mg. CO_2 and 6.500 mg. H_2O		
$C_8H_{19}N$.	Calculated.	C 74.42, H 14.9
129.2	Found.	" 74.12, " 15.1

The substance was converted into the picrate by the procedure previously described.[4] M.p. 132–134°. It had the following composition.

4.504 mg. substance: 7.820 mg. CO_2 and 2.490 mg. H_2O		
$C_{14}H_{22}O_7N_4$.	Calculated.	C 46.92, H 6.19
358.2	Found.	" 47.34, " 6.18

III. N-Dimethylleucinol—The next fraction, after removal of the N-dimethyl-2-amino-4-methylpentane, was collected between 110–120°, giving a substance which had a composition agreeing with that calculated for an N-dimethylaminohexanol.

3.008 mg. substance: 7.302 mg. CO_2 and 3.485 mg. H_2O		
7.709 " " : 0.652 cc. N_2 at 28.5°, *p* 767 mm.		
$C_8H_{19}ON$.	Calculated.	C 66.14, H 13.18, N 9.66
145.2	Found.	" 66.19, " 12.96, " 9.66

This substance formed a picrate which melted at 105–106°, in agreement with the melting point given for the picrate of *dl*-N-dimethylleucinol. It had the following composition.

5.287 mg. substance: 8.700 mg. CO_2 and 2.790 mg. H_2O		
$C_{14}H_{22}O_8N_4$.	Calculated.	C 44.89, H 5.98
374.2	Found.	" 44.87, " 5.90

IV. N,N'-Dimethyl-2,5-Diisobutylpiperazine—After removal of the dimethyl-2-amino-4-methylpentane and the dimethylleucinol the still residue was separated into two fractions, the first one distilling between 120° and 150° at 22 mm. and composed of a mixture of N-dimethylleucinol and N,N'-dimethyldiisobutylpiperazine. The proportion of each in this fraction was calculated from the carbon and hydrogen values.

The second fraction distilled at 150–155° at a pressure of 1 to 2 mm. This substance contained from 72 to 73 per cent carbon, indicating the presence of some dimethylleucinol. Accordingly, a picrate was made of this fraction by dissolving the product in hot

methyl alcohol and adding a hot methyl alcohol solution of picric acid. The picrate was insoluble in the hot methanol, thus separating it from the soluble picrate of dimethylleucinol. The picrate dissolved in hot absolute ethanol and settled out again on the addition of ether. It had a melting point of 255–257° and a composition agreeing with that calculated for the picrate of an N,N′-dimethyldiisobutylpiperazine.

5.910 mg. substance: 9.890 mg. CO_2 and 2.720 mg. H_2O

$C_{14}H_{30}N_2 \cdot C_{12}H_6N_6O_{14}$.	Calculated.	C 45.61, H 5.3
684.3	Found.	" 45.63, " 5.2

This picrate was then decomposed according to the directions given for decomposition of the picrate of N-dimethylleucinol,[4] giving a product which distilled at 80–85° at 0.3 to 0.5 mm. pressure. The substance had a composition agreeing with that calculated for an N,N′-dimethyldibutylpiperazine.

3.303 mg. substance: 9.005 mg. CO_2 and 3.990 mg. H_2O
5.022 " " : 0.549 cc. N_2 at 26°, p 754 mm.
5.311 " " : 5.311 cc. 0.01 N $Na_2S_2O_3$

$C_{14}H_{30}N_2$.	Calculated.	C 74.34,	H 13.4,	N 12.39,	CH_3(N) 13.22
226.3	Found.	" 74.34,	" 13.5,	" 12.38,	" 12.73

Action of Raney's Nickel Catalyst on Dimethylleucinol in Methanol at 185°—Dimethylleucinol (4.5 gm.) was dissolved in 100 cc. of methanol and reduced for 10 hours with 3 gm. of Raney's nickel, at a temperature of 185° and an initial pressure of 2200 pounds. The product was worked up as the free base in the manner described previously. The colorless liquid *completely* distilled at 60–70° under 20 mm. pressure and then redistilled at 110–115° under atmospheric pressure. Yield 2.6 gm.

The picrate of this substance melted at 132–134°. The substance thus had the properties of N-dimethyl-2-amino-4-methylpentane.

Synthesis of N,N′-Dimethylpiperazine by Reduction of Glycylglycine Anhydride—3.0 gm. of glycylglycine anhydride were dissolved in 30 cc. of dry methanol. To the solution were added 5.0 gm. of Raney's catalyst and reduction was carried out at 200° as described. The filtrates from the catalyst and the washings were combined and acidulated with hydrogen chloride gas until acid to Congo red and were then concentrated to a thick syrup.

The residue was taken up in 10 cc. of water and an aqueous solution of sodium picrate was added to it. The yield was nearly quantitative, 9.5 gm. of picrate (1.9 gm. of base). After recrystallization, the melting point was 280°.

The substance had the following composition.

5.822 mg. substance: 8.085 mg. CO_2 and 1.990 mg. H_2O
4.620 " " : 0.797 cc. N_2 at 27°, p 750 mm.

$C_{18}H_{20}O_{14}N_8$.	Calculated.	C 37.75,	H 3.55,	N 19.57
572.2	Found.	" 37.91,	" 3.82,	" 19.37

Synthesis of N,N'-Dimethyl-2,5-Diisobutylpiperazine by Reduction of dl-Leucylleucine Anhydride—3.0 gm. of leucylleucine anhydride and 5 gm. of Raney's catalyst were treated as in the previous experiment. The yield of the picrate was 9.6 gm. (2.82 gm. of base). M.p. 256–258°.

The substance had the following composition.

5.410 mg. substance: 8.995 mg. CO_2 and 2.595 mg. H_2O
5.615 " " : 0.812 cc. N_2 at 26°, p 762 mm.

$C_{26}H_{36}O_{14}N_8$.	Calculated.	C 45.59,	H 5.30,	N 16.35
684.3	Found.	" 45.32,	" 5.36,	" 16.20

Condensation of dl-Leucine Methyl Ester to Its Anhydride in Hydrogenation Apparatus in Absence of Catalyst—This experiment was undertaken to test the hypothesis of piperazine formation from leucine methyl ester through the intermediate formation of ketopiperazine. 4 gm. of *dl*-leucine methyl ester were dissolved in 15 cc. of dry methanol and placed in the hydrogenation apparatus without catalyst for 9 hours at an initial pressure of 1900 pounds per sq. inch and a temperature of 150°. The apparatus was allowed to cool for 10 hours. The partially crystalline reaction product was concentrated under reduced pressure to a white crystalline mass. Yield 2.6 gm.; m.p. 268–270° after recrystallization from 98.5 per cent ethanol.

The substance had the following composition.

4.295 mg. substance: 10.000 mg. CO_2 and 3.790 mg. H_2O
4.300 " " : 0.469 cc. N_2 at 27°, p 759 mm.

$C_{12}H_{22}O_2N_2$.	Calculated.	C 63.66,	H 9.81,	N 12.38
226.2	Found.	" 63.49,	" 9.87,	" 12.38

Condensation of dl-Leucine Methyl Ester to dl-Leucylleucine Anhydride—3 gm. of *dl*-leucine methyl ester were taken up in 5

cc. of dry methanol and heated in a sealed tube at 150° for 9 hours. The partially crystalline reaction product was concentrated under reduced pressure to a crystalline mass. Yield 2.0 gm.; m.p. 268–270° after recrystallization from ethanol.

The substance had the following composition.

4.926 mg. substance:	11.505 mg. CO_2 and 4.300 mg. H_2O		
4.480 " "	: 0.484 cc. N_2 at 24°, p 759 mm.		
	$C_{12}H_{22}O_2N_2$.	Calculated.	C 63.66, H 9.81, N 12.38
	226.2	Found.	" 63.69, " 9.76, " 12.40

In a similar way 1.0 gm. of *l*-leucine methyl ester, $[\alpha]_D^{26} = +16.7°$, yielded 0.7 gm. of the active ketopiperazine, m.p. 271°.

4.313 mg. substance:	10.100 mg. CO_2 and 3.790 mg. H_2O		
4.600 " "	: 0.502 cc. N_2 at 26°, p 759 mm.		
	$C_{12}H_{22}O_2N_2$.	Calculated.	C 63.66, H 9.81, N 12.38
	226.2	Found.	" 63.85, " 9.90, " 12.48

$$[\alpha]_D^{26} = \frac{-0.45° \times 100}{1 \times 16} = -2.81° \text{ (in methanol)}$$

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THE VERATRINE ALKALOIDS

VII. ON DECEVINIC ACID

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Until recently the study of degradation products from cevine has been confined to the mixtures obtained by pyrolytic procedures such as selenium dehydrogenation and soda lime distillation. However, in a recent (1) short communication the isolation was recorded of a non-nitrogenous acid, $C_{14}H_{14}O_6$, which resulted in appreciable yield by the oxidation of cevine with chromic acid. The study of this acid has now been carried further and, while confirming this formulation, has given information which affords a suggestion of its general nature. For convenience the designation *decevinic acid* has been adopted for this acid.

On being heated with sulfur at about 300°, decevinic acid was rapidly dehydrogenated and from the melt a crystalline substance was isolated (melting at 245–246°) which appeared to be the major product and analysis of which agreed with a formula $C_{12}H_6O_4$. The formulation of the substance, its behavior towards alkali (2 moles were required), the phenolic color reactions exhibited by it, and its melting point suggested at once 2-hydroxy-1,8-naphthalic anhydride (I) which has already been described (2) with the melting point 245–246°. After saponification with stronger alkali it was degraded with loss of CO_2 to a monobasic acid which corresponded in properties with those recorded for 2-hydroxy-8-naphthoic acid (2). The identity of the hydroxyanhydride, $C_{12}H_6O_4$, was confirmed by methylation to a monomethyl ether, $C_{13}H_8O_4$, which was shown by comparison in properties and mixed melting points to be identical with 2-methoxynaphthalic anhydride (2, 3). The latter was obtained by oxidation of 3-methoxyacenaphthenequinone (4). These observations,

barring rearrangements, at once indicate that decevinic acid must possess a hydronaphthalene ring system which in turn constitutes a portion of the cevine molecule. A study of the behavior of this acid towards alkali and also on acetylation and methylation has given information from which certain tentative conclusions may be drawn.

As previously reported (1), titration of decevinic acid in the cold with alkali has shown the presence of two acid groups or of an acid and a labile lactone group. When the acid was refluxed with excess 0.1 N alkali, no appreciable additional alkali was consumed. However, with N alkali an additional equivalent was required with degradation to the acid to be described below. With diazomethane decevinic acid yields, as reported, a dimethyl derivative. More recently with acetic anhydride an *acetyldecevinic acid* has been prepared. The latter on methylation gave an *acetyldecevinic methyl ester* which proved to be a monomethyl ester. Since the above dimethyl derivative could not be acetylated, the acetyl group must be assumed to occupy the same position as one of the methyl groups. The dimethyl derivative on careful titration in the cold consumed only 1 equivalent of alkali but when heated with 0.1 N alkali an extra equivalent was required. In the former case partial saponification occurred, since the product which was isolated proved to be a *monomethyl derivative* of decevinic acid. Like the original dimethyl derivative, this also could not be acetylated.

If the above acetyldecevinic methyl ester was refluxed with methyl alcohol, the acetyl group was removed with production of a *decevinic monomethyl ester* isomeric with the above monomethyl derivative. Contrary to the latter, its methyl group was so labile towards alkali that it behaved on direct titration like decevinic acid itself and consumed almost directly 2 equivalents of alkali. It appears, therefore, that acetylation must occur at the same point as that occupied by the more stable methyl group.

Two possible interpretations can be considered to explain these observations. Decevinic acid may be a dibasic acid in which one carboxyl group forms a more stable ester than the other. To explain the failure of the dimethyl or stable monomethyl derivative to acetylate, it would be necessary to assume that such acetylation can occur only on the carboxyl group which forms the

stable ester group; in other words, production of a mixed anhydride. Otherwise, decevinic acid must be a monobasic lactone acid containing a labile lactone group and also an enolic hydroxyl group which is the point of acetylation as well as of methylation. The latter alternative fits in best with a number of observations which have been made. The characteristic color reactions given with ferric chloride support the presence of an enolic hydroxyl group.

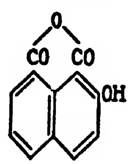
Of importance is the ready degradation of decevinic acid with alkali. Although under gentle conditions 2 equivalents of alkali are consumed, under more vigorous conditions a 3rd equivalent is used up and CO_2 is split off with simultaneous addition of a mole of H_2O with the production of *an acid*, $C_{13}H_{16}O_5$, which no longer gives the prompt typical reaction with ferric chloride. This acid titrates as a dibasic acid (or monobasic lactone acid) and forms a *dimethyl derivative*. No other characteristic derivative was obtained from this acid. However, when distilled or heated with alkali, further degradation occurred with CO_2 cleavage to a *keto-lactone*, $C_{12}H_{16}O_3$. This substance although neutral to sodium carbonate solution consumed 1 equivalent of alkali owing to cleavage of a lactone group. The presence of a carbonyl group was shown by the formation of both an *oxime* and a *phenylhydrazone*.

This series of degradations when considered with the production of a naphthalic anhydride derivative on dehydrogenation places limitations on the positions which can be assigned to the remaining carbon atoms other than the 1 and 8 carbon atoms of hydroxynaphthalic anhydride. Apparently, one of these must be an angular methyl group situated on a carbon atom common to both rings. The 2nd carbon atom could be attached either directly to the ring system as a carboxyl (a lactone) carbon atom or to a side chain from which one of the carboxyl groups of hydroxynaphthalic anhydride arises. In the latter case, this requirement would be met by an α-keto acid (or lactone) side chain.

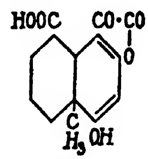
The behavior of decevinic acid towards alkali is perhaps more easily explained by the first assumption. In this case it would have to be a dibasic lactone acid. The fact that it requires more vigorous treatment with alkali for the consumption of a 3rd equivalent of alkali which involved loss of CO_2 can be due either to

resistance to saponification or to rapid relactonization during titration under the gentler conditions. The production of the $C_{13}H_{16}O_5$ derivative and in turn the ketolactone, $C_{12}H_{16}O_3$, can be simply explained by successive loss of carboxyl groups and final relactonization presumably on a double bond. There remain, however, certain observations which are difficult to harmonize with this interpretation. Among these is the fact that the condensation product with *o*-phenylenediamine described below must involve the lactone group and, if a glyoxaline derivative, would still be a dibasic acid, which is contrary to our experience.

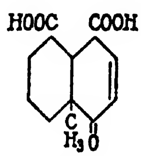
A second possibility is the formula (II) of an α-ketolactone acid. This structure was first suggested by the fact that decevinic acid



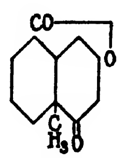
I



II



III



IV

was found to react readily with *o*-phenylenediamine to give in good yield a *condensation product*, $C_{20}H_{20}O_5N_2$, involving the loss of only 1 mole of water and which on titration behaved as a monobasic acid. If this product is a hydroxyquinoxaline derivative (or even a glyoxaline derivative) arising in a normal manner from the condensation with the α-ketolactone group and simultaneous opening of the latter, only 1 mole of water would be lost. Such a condensation product would still be a monobasic acid. The conversion of such an α-ketolactone acid on saponification with loss of CO_2 and addition of water would require certain involved rearrangements in order to lead to a structure (III) for the dibasic acid $C_{13}H_{16}O_5$. The latter on decarboxylation and lactonization could then give a structure (IV) for the ketolactone, $C_{12}H_{16}O_3$.

Such interpretations can be at the moment only tentative and it is hoped to obtain other clarifying evidence.

Hydrogenation studies with this series of substances, although still incomplete, have given results which will have to be considered in the final interpretation of their nature. Decevinic acid on hydrogenation yielded a mixture which could not be directly crystallized. After low pressure distillation, however, accompanied apparently by water cleavage, a substance was obtained which after recrystallization gave analytical figures agreeing with a formulation $C_{14}H_{20}O_4$. Although on direct titration 2 equivalents of alkali were consumed, it gave only a *monomethyl ester*, $C_{15}H_{22}O_4$. The acid must therefore be a *monobasic lactone acid*.

The above ketolactone, $C_{12}H_{16}O_3$, on hydrogenation yielded a mixture from which two substances were isolated for which the provisional formulas $C_{12}H_{18}O_2$ and $C_{12}H_{18}O_3$ have been derived. The former was produced presumably by reductive cleavage of the lactone group to the desoxy acid and subsequent relactonization on the hydroxyl group produced by reduction of the carbonyl group. The substance $C_{12}H_{18}O_3$ represents possibly an intermediate step.

The above observations are by no means sufficient to permit final conclusions as to the structure of decevinic acid. The possible origin of a hydronaphthalene derivative from the ring system of cevine will be considered elsewhere in the discussion of results obtained in the degradation of this alkaloid by other methods.

EXPERIMENTAL

Dehydrogenation of Decevinic Acid—A well ground mixture of 1 gm. of decevinic acid and 0.23 gm. of sulfur was placed in a test-tube with the upper end sealed to a smaller glass tube to act as an air condenser. Before heating, the air was replaced by nitrogen. The temperature of the salt bath was slowly raised until gases were evolved. This occurred suddenly at about 300° and was rapidly ended. After 5 minutes heating was interrupted. 200 cc. of gas were evolved. Nothing appeared to condense in the tube above the reaction mixture with the exception of a small amount of crystalline material just above the melt. This suggested absence of water formation during the dehydrogenation.

The melt was broken up and extracted with boiling acetone. This extract after evaporation was fractionated in a molecular still. 0.32 gm. of material distilled up to an oil bath temperature of 150° under a pressure of 0.001 mm. or less. Upon recrystallization from acetone 0.145 gm. of yellow needles was obtained which had a micromelting point of 245–246°. Dziewoński and Koewa (2) reported 245–246° for 2-hydroxynaphthalic anhydride.

$C_{12}H_6O_4$. Calculated, C 67.28, H 2.82; found, C 67.45, H 3.11
" " 67.35, " 2.99

10.57 mg. of substance suspended in alcohol were titrated against phenolphthalein with 0.1 N NaOH. After the material was heated with a slight excess of alkali and titrated back, a total of 1.108 cc. was required. The end-point was not easily seen because of the color of the substance itself; calculated for 2 equivalents, 0.987 cc.

The substance gave a red color in alcoholic solution with ferric chloride and coupled with diazotized sulfanilic acid to give a red color.

0.075 gm. of this anhydride was heated in a solution of 0.3 gm. of NaOH in 1 cc. of water for 1.5 hours on the steam bath in an atmosphere of hydrogen. The acid which precipitated on acidification was extracted with ether and recrystallized twice from dilute ethyl alcohol with bone-black. The resulting substance melted at 257–259°. Dziewoński and Koewa (2) reported a melting point of 257° for 2-hydroxy-8-naphthoic acid.

$C_{11}H_8O_3$. Calculated, C 70.22, H 4.28; found, C 69.95, H 4.54

The substance gave a color with $FeCl_3$ and coupled with diazotized sulfanilic acid.

8.967 mg. of substance dissolved in alcohol were titrated against phenolphthalein with 0.1 N NaOH; found, 0.571 cc.; calculated for 1 equivalent, 0.477 cc. On being heated with excess alkali, no additional alkali was consumed.

60 mg. of the above anhydride were treated in acetone with excess diazomethane. Long slender needles were obtained from acetone, which melted at 256–257°.

$C_{13}H_8O_4$.	Calculated.	C 68.42,	H 3.53,	OCH_3	13.60
	Found.	" 68.35,	" 3.70,	"	13.13

This no longer gave a color with $FeCl_3$ and did not couple with diazotized sulfanilic acid. A mixed melting point with synthetic 2-methoxynaphthalic anhydride showed no depression and it corresponded in other properties with the synthetic material. The latter was prepared by oxidation with $KMnO_4$ in acetone solution of 3-methoxyacenaphthenequinone which was obtained according to the directions of Staudinger, Goldstein, and Schlenker (4). The anhydride was recrystallized from acetone and melted at 256–257°. Dziewoński and Koewa (2) and Davies, Heilbron, and Irving (3) reported a melting point of 255°.

$C_{13}H_8O_4$. Calculated, C 68.42, H 3.52; found, C 68.51, H 3.33

Decevinic Acid—Since the details of the method of preparation and properties of this substance and its ester could not be recorded in the earlier communication (1), they are now given in detail.

20 gm. of recrystallized cevine were dissolved in a mixture of 200 cc. of H_2SO_4 (1.84) and 800 cc. of water. 100 gm. of CrO_3 were then carefully added with cooling to avoid appreciable rise in temperature. Vigorous evolution of gas occurred during the addition of the reagent. After standing at room temperature for an hour, the solution was refluxed for an hour. It was then cooled and the excess of chromic acid was reduced with hydrazine hydrate. The mixture was then continuously extracted with ether overnight and the resulting ether extract was decanted from a small amount of a green solid deposit. It was dried over anhydrous $MgSO_4$ and the solvent was boiled off. The colorless liquid residue weighed approximately 10 gm. It was water-soluble and of acid character. Since repeated efforts to obtain crystalline material directly from this liquid were unsuccessful, it was treated as follows:

The mixture was heated in a flask through which a current of N was passed for an hour in an oil bath held at a temperature of 180°. During this heating crystalline material appeared in the melt and slow evolution of a gas was apparent. The melt was then treated with 4 volumes of ether and, after trituration in order to dissolve sticky material, the crystalline residue was collected with a little ether. It weighed 2 gm. Upon recrystallization from acetone the product melted at 273–278° with decomposition.

$[\alpha]^{25}_D = +47.6°$ (c = 0.925 in pyridine)

$C_{14}H_{14}O_6$. Calculated, C 60.41, H 5.07; found, C 60.51, H 5.20
" " 60.45, " 5.14

0.0150 gm. of substance in 2.5 cc. of ethyl alcohol was titrated against phenolphthalein with 0.1 N NaOH; found, 1.114 cc.; *calculated for 2 equivalents, 1.082 cc.* No appreciable additional *alkali was consumed after the substance was boiled for 2 hours with excess 0.1* N *alkali.*

0.200 gm. *of substance was suspended in 1 equivalent of* N NaOH. The end-point to phenolphthalein was reached only after addition of a 2nd equivalent or a total of 1.44 cc. of NaOH. Solution was just complete at this point. More alkali was then added until a total of 4.00 cc. had been reached. The solution was heated on the steam bath in an atmosphere of hydrogen for 1 hour and then titrated back with N HCl. Inclusive of the above 1.44 cc., a total of 2.08 cc. of NaOH was consumed; calculated for 3 equivalents, 2.16 cc. The substance, $C_{12}H_{16}O_3$, reported below, could readily be isolated from the hydrolysate.

The Methyl Ester, $C_{16}H_{18}O_6$—0.1 gm. of decevinic acid was dissolved in acetone and treated with an excess of diazomethane. Addition of ether to the concentrated solution gave a slight turbidity followed by crystallization. 70 mg. of rhombic crystals were obtained which melted at 165–166°.

$C_{16}H_{18}O_6$.	Calculated.	C 62.75,	H 5.92,	OCH_3	20.26
	Found.	" 62.96,	" 6.07,	"	19.90
	"	" 62.73,	" 6.01		

The molecular weight determined by the Rast method was found to be 326; calculated, 306.14.

0.0149 gm. of the ester in 3 cc. of ethyl alcohol was titrated against phenolphthalein with 0.1 N NaOH; found, 0.478 cc.; calculated for 1 equivalent, 0.483 cc. After addition of excess 0.1 N NaOH and after refluxing for 2.5 hours an additional 0.437 cc. was consumed. The end-point was not very sharp because of an interfering color. This titration behavior was a little different from the titration in aqueous N NaOH.

0.200 gm. of the dimethyl ester was treated directly in the cold with N NaOH. As more NaOH was added, it dissolved but a point alkaline to phenolphthalein was not reached until 0.660

cc. had been added; calculated for 1 equivalent, 0.654 cc. All the ester was in solution at this point. When excess NaOH was added and the solution was heated on the steam bath for 1 hour, the alkaline decomposition reported below occurred with formation of the substance $C_{12}H_{16}O_3$.

Acetyldecevinic Acid—0.4 gm. of decevinic acid was refluxed with 10 cc. of acetic anhydride for 1 hour. After removal of excess reagent with toluene the residue was crystallized from acetone. 0.3 gm. of flat needles was obtained which melted at 169–171° with previous sintering.

The substance is soluble in dilute carbonate in the cold and gives a strong test with ferric chloride when dissolved in ethyl alcohol.

$C_{16}H_{16}O_7$. Calculated, C 59.98, H 5.04; found, C 60.24, H 5.11

0.117 gm. of the substance was titrated directly against phenolphthalein with 1.1 N NaOH. 0.980 cc. was required; calculated for 3 equivalents, 0.996 cc. After addition of excess N alkali and heating for an hour, an additional mole was consumed as in previous cases.

Acetyldecevinic Methyl Ester—0.1 gm. of the above acetyl derivative was esterified with diazomethane in acetone. It crystallized readily on addition of ether to the concentrated solution. 0.06 gm. of leaflets was obtained which melted at 182–183°.

$C_{17}H_{18}O_7$. Calculated.	C 61.06,	H 5.43,	OCH_3	9.28
Found.	" 61.03,	" 5.49,	"	9.11

0.0049 gm. of substance on direct titration with 1.01 N NaOH against phenolphthalein required 0.0410 cc.; calculated for 3 equivalents, 0.0436 cc.

Decevinic Methyl Ester—0.06 gm. of the above acetyl derivative was refluxed in 2 cc. of methyl alcohol for 2 hours. The solution crystallized on concentration. 0.021 gm. of the ester was collected which melted at 242–245°.

$C_{15}H_{16}O_6$. Calculated.	C 61.62,	H 5.52,	OCH_3	10.60
Found.	" 61.27,	" 5.60,	"	7.25

0.0049 gm. when titrated against phenolphthalein with 1.01 N NaOH required 0.0315 cc.; calculated for 2 equivalents, 0.0332 cc.

Partial Hydrolysis of the Ester, $C_{16}H_{18}O_6$—0.1 gm. of the ester was treated with 0.300 cc. of 1.1 N NaOH and the mixture was slightly warmed until just all in solution. It was treated with a slight excess of HCl and the material which separated was extracted with ether. The ether solution was dried over anhydrous $MgSO_4$, and then concentrated somewhat when crystallization occurred. 70 mg. of substance were collected which melted at 128°.

$C_{16}H_{18}O_6$.	Calculated.	C 61.62, H 5.52, OCH_3 10.61
	Found.	" 61.88, " 5.51, " 10.29

0.082 gm. of substance on direct titration with 1.1 N NaOH consumed 0.285 cc.; calculated for 1 equivalent, 0.255 cc. Boiling with acetic anhydride failed to form an acetyl derivative of this substance.

Condensation of o-Phenylenediamine with Decevinic Acid—1 gm. of decevinic acid and 0.78 gm. of *o*-phenylenediamine were dissolved in sufficient hot methyl alcohol. The methyl alcohol was then boiled off and the residue was heated on the steam bath for 1 hour. The crystalline material was recrystallized from a large volume of methyl alcohol. 0.48 gm. of yellow flat columns was obtained which melted at 300–302°. It was soluble in dilute carbonate and did not give a color test with ferric chloride.

$C_{20}H_{20}O_5N_2$.	Calculated.	C 65.20, H 5.47, N 7.60
	Found.	" 65.44, " 5.49, " 7.85

0.125 gm. of substance on direct titration with 1.1 N NaOH required 0.315 cc.; calculated for 1 equivalent, 0.309 cc. Excess alkali was then added and the solution was heated for an hour on the steam bath. 0.542 cc. more of alkali was consumed. The end-point in this case was not sharp owing to interfering color.

Alkaline Degradation of Decevinic Acid. The Acid, $C_{13}H_{16}O_5$—2 gm. of decevinic acid were treated with a solution of 6.4 gm. of NaOH in 20 cc. of water. The solution was allowed to stand at room temperature for 2 hours and then acidified with HCl. CO_2 was evolved. The insoluble material which separated was extracted with ether. The dried ether extract gave, on concentration, a residue which was crystallized from acetone-ether. 0.43 gm. of substance was obtained. After recrystallization it melted at 150–155° with effervescence.

$C_{13}H_{16}O_5$. Calculated, C 61.88, H 6.39; found, C 61.92, H 6.54

The substance dissolved in alcohol did not give a color test with ferric chloride.

13.410 mg. of substance when titrated against phenolphthalein with 0.1 N NaOH consumed 1.057 cc. After addition of excess alkali and heating no more alkali was consumed; calculated for 2 equivalents, 1.064 cc.

The Dimethyl Ester, $C_{15}H_{20}O_5$—0.1 gm. of the above acid was esterified in acetone with diazomethane. The ester could not be crystallized and was distilled in a high vacuum. The distillate which was a clear viscous oil was analyzed as such.

$C_{15}H_{20}O_5$.	Calculated.	C 64.24, H 7.19, OCH_3 22.13
	Found.	" 63.43, " 7.12, " 21.20

The Ketolactone, $C_{12}H_{16}O_3$—0.1 gm. of the above acid, $C_{13}H_{16}O_5$, was placed in a small sublimation apparatus under 0.1 mm. pressure. When a temperature of 180° was reached, a sticky viscous resin distilled. This was accompanied by gas evolution as evidenced by a fall in the pressure. The distillate crystallized readily from ether. After recrystallization from acetone it melted at 165–168°. $[\alpha]_D^{25} = -50°$ (c = 0.99 in chloroform).

$C_{12}H_{16}O_3$. Calculated, C 69.19, H 7.75; found, C 69.33, H 7.88

The substance was not soluble in sodium carbonate solution and the solution in alcohol did not give a color test with ferric chloride.

14.497 mg. of substance on direct titration against phenolphthalein with 0.1 N NaOH consumed 0.745 cc.; calculated for 1 equivalent, 0.697 cc. After addition of excess alkali and heating, no more alkali was consumed.

The substance did not react with diazomethane in acetone solution.

The Phenylhydrazone—0.025 gm. of the above ketolactone was treated with 0.05 gm. of phenylhydrazine and warmed a moment on the steam bath. The resulting melt was crystallized from ethyl alcohol. 0.01 gm. of needles was obtained which melted at 175–178°.

$C_{18}H_{22}O_2N_2$. Calculated, C 72.46, H 7.43; found, C 72.35, H 7.40

The Oxime—0.03 gm. of the ketolactone in 5 cc. of ethyl alcohol was treated with a solution of 0.1 gm. of hydroxylamine hydrochloride dissolved in 1.2 cc. of 1.1 N NaOH. The solution was concentrated on the steam bath. On cooling, needles separated which after collection with water melted at 194–195° with previous sintering.

$C_{12}H_{17}O_3N$. Calculated, C 64.57, H 7.96; found, C 64.67, H 7.61

Catalytic Hydrogenation of Decevinic Acid—0.5 gm. of the acid suspended in 40 cc. of alcohol was shaken with 0.15 gm. of the platinum oxide catalyst of Adams and Shriner under a hydrogen pressure of about 3 atmospheres. After 24 hours no more hydrogen was absorbed and all the substance was dissolved. Approximately 4.7 moles of hydrogen were absorbed. After removal of the catalyst and evaporation of the solvent the residue could not be induced to crystallize. It was therefore placed in a molecular still. As distillation was effected, a gas appeared to be split off from the material, since pressures under 0.01 mm. could not be maintained during the process. 0.33 gm. distilled up to an oil bath temperature of 170°. The distillate crystallized from ether and yielded 0.13 gm. of substance which melted at 205–230°. After two recrystallizations from acetone 0.055 gm. of long narrow plates was obtained which melted at 237–239°. The melting point did not change on further recrystallization.

$C_{14}H_{20}O_4$.	Calculated, C 66.64, H 7.99;	found, C 66.94, H 8.12
		" " 66.64, " 7.58
		" " 66.99, " 7.74

0.0079 gm. of substance when titrated against phenolphthalein with 1.01 N NaOH required 0.0610 cc.; calculated for 2 equivalents, 0.0621 cc.

The Methyl Ester—0.03 gm. of the above acid was esterified in acetone with diazomethane. The solution upon evaporation yielded a residue which crystallized from ether. 0.015 gm. of needles was collected which melted at 127–128°. The melting point was unchanged by further recrystallization.

$C_{15}H_{22}O_4$.	Calculated.	C 67.62,	H 8.33,	OCH_3	11.65
	Found.	" 67.82,	" 8.26,	"	11.72
	"	" 67.91,	" 7.64		
	"	" 67.76,	" 8.04		

Hydrogenation of the Ketolactone, $C_{12}H_{16}O_3$—0.1 gm. of substance was hydrogenated with 0.05 gm. of platinum oxide catalyst in 3 cc. of alcohol, under 3 atmospheres pressure. After 1 hour absorption was completed at approximately 1.7 moles of hydrogen above that required by catalyst. The residue obtained after removal of the solvent crystallized nicely from ether-petroleum ether mixture but the crystals did not appear to be homogeneous and melted at 80–90°. The analytical data suggested loss of oxygen as well as hydrogenation. Since fractional crystallization did not appear to yield a homogeneous product, fractional distillation was attempted.

A second run of 0.1 gm. was combined with the first and the resulting material amounting to 0.18 gm. was fractionated in a small still (5 cm. column) of the type reported previously (5). Six approximately equal fractions were collected under 0.1 mm. pressure. The first fraction was crystalline and melted at 90–91°; found, C 73.68, H 9.30. The last fraction was semiliquid; found, C 69.96, H 9.39.

The first and second fractions were combined and recrystallized from ether. Heavy columns were obtained which melted at 97°.

$C_{12}H_{18}O_2$. Calculated, C 74.19, H 9.35; found, C 74.50, H 9.23

The final fraction was recrystallized from ether. It melted at 65–73° with a few crystals persisting up to 90°. This was analyzed directly, since there was not sufficient of this material for both recrystallization and analysis.

$C_{12}H_{18}O_3$. Calculated, C 68.54, H 8.63; found, C 69.09, H 8.72

BIBLIOGRAPHY

1. Craig, L. C., and Jacobs, W. A., *J. Am. Chem. Soc.*, **61**, 2252 (1939).
2. Dziewoński, K., and Koewa, A., *Chem. Zentr.*, **1**, 649 (1929).
3. Davies, R. I., Heilbron, I. M., and Irving, F., *J. Chem. Soc.*, 2715 (1932).
4. Staudinger, H., Goldstein, H., and Schlenker, E., *Helv. chim. acta*, **4**, 349 (1921).
5. Craig, L. C., *Ind. and Eng. Chem., Anal. Ed.*, **9**, 441 (1937).

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THE INTERPRETATION OF SIMPLE ELECTROPHORETIC PATTERNS

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Introduction.—An electrophoresis experiment with a single protein may be represented diagrammatically as follows. Initially boundaries are formed at the level a′-a, of Fig. 1, in the two sides of the cell, between the protein solution P and the buffer solution B. After displacement of the boundaries from behind the horizontal glass plates of the cell, passage of a current causes one of the boundaries, a, to descend through a volume V_d to a new position d, as in Fig. 2, and the other, a′, to rise through a volume V_r to the position r of the same figure. Under ideal conditions, approximated with a dilute solution of a protein in a buffer of sufficient capacity, the volumes V_d and V_r are equal and a knowledge of their value permits a precise computation of the mobility of the protein. In general, however, the volume V_r is greater than V_d and an analysis of the concentration changes resulting from the electrolysis is necessary in order to obtain mobilities from the experimental results. It is the purpose of this paper to describe electrophoretic experiments on ovalbumin, at 0°, in which these concentration changes have been determined, and to outline an analysis of the data for the purpose of obtaining accurate mobilities.

Methods.—The recent development of methods for studying and recording refractive index gradients, initiated by Tiselius,[1] has made possible the precise study of electrophoretic boundaries. From "electrophoretic patterns" thus obtained concentration changes during electrophoresis may be computed. The available procedures are the scale method of Lamm,[2] the diagonal schlieren method of Philpot[3] and Svensson[4] and the schlieren scanning method developed in this Laboratory[5,6] and used in the present research.

(1) Tiselius, *Trans. Faraday Soc.*, **33**, 524 (1937).
(2) Lamm, *Nova Acta Reg. Soc. Sci. Upsaliensis*, Series IV, **10**, No. 6 (1937).
(3) Philpot, *Nature*, **141**, 283 (1938).
(4) Svensson, *Kolloid-Z.*, **87**, 181 (1939).
(5) Longsworth, THIS JOURNAL, **61**, 529 (1939).
(6) Longsworth, Shedlovsky and MacInnes, *J. Exptl. Med.*, **70**, 399 (1939).

In outline the schlieren scanning method is illustrated in Fig. 3. An image at P of the illuminated slit S-S is formed by the schlieren lens D. The camera lens O is focused on the electrophoresis cell E and forms an image (full sized in our apparatus) on the screen at G-G. Now if the fluid in the cell is homogeneous this image will be uniformly illuminated. On

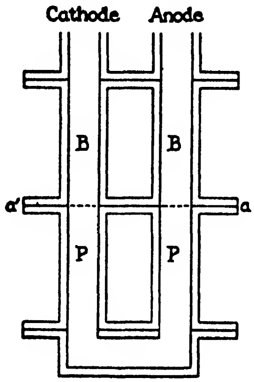

FIG. 1.

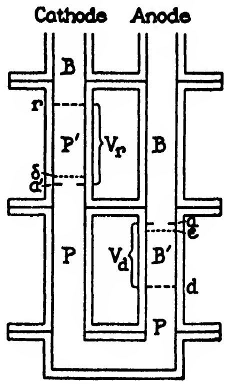

FIG. 2.

FIGS. 1 and 2. Diagrams of the electrophoresis cell illustrating the formation and relative position of the boundaries.

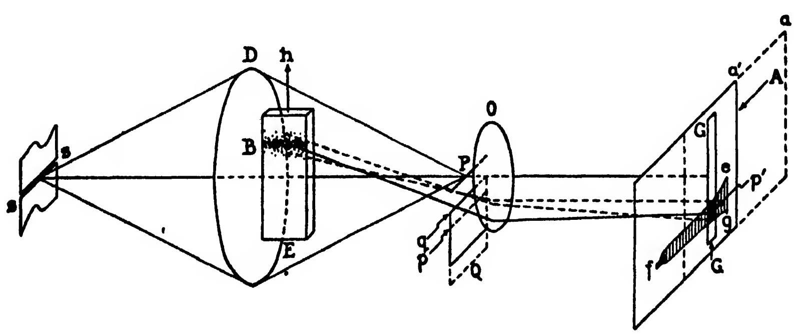

FIG. 3. Diagram of the schlieren scanning method for the photographic recording of gradients of refractive index.

the other hand, if there is a boundary, B, between, for instance, a protein-bearing solution and a buffer, there will be a region in which the refractive index varies with the height in the cell, and light which would normally pass to P is deflected downward, since the solution with the greater refractive index is on the bottom. If the opaque schlieren diaphragm Q is raised to a point p where it intercepts the most deflected light, a dark band will appear on the screen, conjugate to the region of steepest gradient in the boundary B. Such a band appears in the image G-G at p′.

However, a boundary between two solutions does not consist of a single geometric plane, but of a region in which the composition varies from that of one solution to that of the other. The refractive index, n, in such a region changes continuously with the height, h, of the liquid in the cell. The gradient, dn/dh, of refractive index, for each boundary, will thus, theoretically at least, vary from zero to a maximum, and back to zero. In Fig. 3 the variation of the gradient, dn/dh, of the boundary B in the cell E, is represented by the density of the shading. The pencil of light passing through the layer having the maximum value of the gradient will be most bent from the normal path and will be the first to be intercepted when the schlieren diaphragm Q is raised. On lifting this diaphragm still further, *i.e.*, to the level q, less refracted pencils of light will also be intercepted and the schlieren band in the image G-G will widen. This obviously can be continued until the whole field has been covered. In the schlieren scanning method this process is made continuous and is recorded photographically.. The image of the cell at G-G is masked by a narrow vertical slit and a photographic plate A is moved in the direction of the arrow at a constant rate across this slit. Actuated by the same mechanism, the schlieren diaphragm Q is given a steady movement upward. The resulting (positive) photographic record for a typical single boundary is indicated by the area e-f-g. The displacement of the diaphragm Q from the position P is proportional to the gradient at levels in the cell E conjugate to the edges of the schlieren bands. Thus the contour of the area e-f-g indicates both the position and the magnitude, of the refractive index gradients existing in the boundary. Since the photographic plate A was in position a at the time the schlieren diaphragm was at p, a section of the band p′ appears at f when the plate has been moved to a′. It will be seen that the usual schlieren bands are narrow sections through the area e-f-g. By a system of gears the ratio of the rates of motion of the schlieren diaphragm Q to that of the plate A is given a constant value, such as one to three, and this ratio can be varied by changing the gears. For establishing the base line of the schlieren patterns the position of the diaphragm Q may be read accurately with a micrometer.

Experimental Results and Discussion.—The type of phenomenon to be discussed in this paper is illustrated in Fig. 4, which represents electrophoretic patterns of a 1.36% solution of ovalbumin at *p*H 3.92 in a 0.1 normal sodium acetate buffer.[7]

(7) The ovalbumin used in this research was prepared from fresh hen's eggs. Although three times recrystallized, it was not electrophoretically homogeneous. At most *p*H values the main component is accompanied by a second component migrating

In order to interpret these patterns in terms of concentrations and mobilities, information must be available about (a) the base line of the diagram and (b) the starting position of the boundaries. The base line is obtained as shown at c-c′ of Fig. 4 by a *scanning exposure of the cell immediately after the formation of the boundaries but before the latter have been shifted into view from behind the horizontal glass plates.* The procedure consists in setting the schlieren diaphragm, Q of Fig. 3, just below P, the exact position of the diaphragm being given by the reading, m of a micrometer, and then scanning until the diaphragm is somewhat above P. The lower edges of the strips c and c′ of Fig. 4 are thus the schlieren patterns of the windows of the cell and thermostat, deviations of these edges from the horizontal indicating imperfections in the windows. The upper edges of the strips c and c′ correspond to a reading, m, of the micrometer. The equivalent position on the patterns a and b, Fig. 4, is at m-m. Consequently when the upper edges of the strips c-c′ are brought into coincidence with the line m-m the lower edges of these strips constitute the base line for the patterns. In order to obtain the position of the freshly formed boundaries after they have been shifted into view, the schlieren bands shown at d-d′ in Fig. 4 are recorded on the photographic plate. As Guggenheim[8] has suggested, the diffusion process smooths out the slight irregularities in the distribution of concentrations resulting from the formation and shifting of the boundaries. As illustrated by the finite width of the bands in the strip d-d′, the boundaries are not infinitely sharp. However, the positions of the mid-points of these bands may be taken as the positions at zero time from which mobilities can be computed.

It will be seen in Fig. 4 that two peaks or maxima are evident in the patterns of both the rising and descending boundaries but that the patterns are far from being mirror images of each other. This may, possibly, be an extreme case but is all the more useful in illustrating deviations from ideal behavior and in testing the validity of the interpretation to be given below.

The deviations, or so-called "boundary anomalies," illustrated by these patterns are as follows. (1) The rising boundary r is much sharper than that, d, descending into the protein solution. (2) The boundary r has swept through a larger volume V_r, than that, V_d, of the boundary d. (3) The total shaded area of Fig. 4a is equal to that of Fig. 4b, but the partial area A_r under the rising boundary r is less than that, A_d, for the descending boundary d. Since these areas, as will be shown, are proportional to the concentration changes at the boundaries it is apparent

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somewhat more slowly, as shown in Fig. 2 of reference (5) and in Fig. 6 of this paper. Similar patterns have been obtained with samples of ovalbumin from four different laboratories. A study of egg white now in progress indicates that the slow component is present in the starting material. Ovalbumin appears homogeneous in the patterns reproduced in Fig. 4, because the *p*H-mobility curves of the two components apparently intersect in the neighborhood of the isoelectric point. At *p*H 3.92 the protein may be considered electrically homogeneous, thereby simplifying the interpretation of the patterns.

(8) Guggenheim, This Journal, 52, 1315 (1930).

that the change is less at the rising boundary than at the descending one. Also (4), the concentration distribution in a boundary is not, in general, symmetrical about the maximum as is shown by the shapes of the peaks d and r.

These deviations were explained qualitatively by the authors[9] as follows. In practice the protein solution is prepared by dialysis against a buffer solution. A difference, due to the Donnan equilibrium, of salt composition exists between the two solutions when dialysis is complete. Moreover, the protein solution contains conducting constituents, the protein ions, that

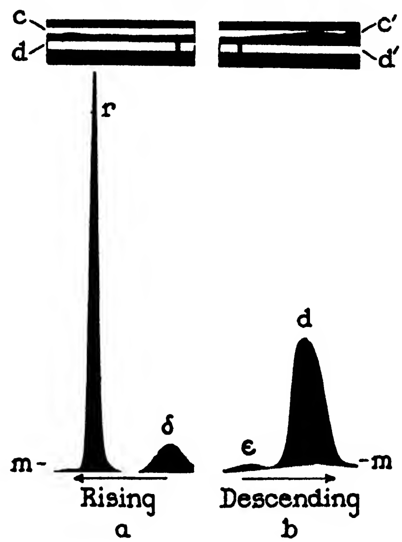

FIG. 4. Electrophoretic patterns of a 1.36% solution of ovalbumin in a 0.1 *N* sodium acetate buffer at *p*H 3.92 and 0°. Scanning exposure made after electrolysis for 13,000 seconds at 5.39 volts per cm.

are not present in the buffer solution. When passage of a current causes a boundary between two such solutions to move from a to d of Fig. 2, for example, there is found in the intervening volume V_d a buffer solution of composition [B′]. This composition has been adjusted, in general, to a value different from that of B.[10] The boundary ε, which moves very slowly,

(9) Longsworth and MacInnes, *Chem. Rev.*, **24**, 271 (1939).

(10) This adjustment is understood for some simple cases (Kohlrausch, *Ann. Physik.*, **62**, 209 (1897); Weber, "Die partiellen Differential-Gleichungen der mathematischen Physik," 5te Aufl., Braunschweig, 1910, **1**, 195-203, 503-527; Longsworth, THIS JOURNAL, **52**, 1897 (1930); Henry and Brittain, *Trans. Faraday Soc.*, **29**, 798 (1933)) but the theory for the more complicated systems frequently encountered in practice has not been developed.

thus forms between two solutions of the same buffer, but at different concentrations,[11] and is quite evident in Fig. 4. With the boundary r moving upward into the buffer solution, there is a similar but more complicated adjustment of the composition of the protein solution which replaces the buffer as the boundary rises. The resulting concentration boundary δ, between the solutions P and P′, Fig. 2, also moves very slowly under the influence of the current. The δ boundary, also shown in Fig. 4, is much more visible than the ϵ boundary because the former involves a gradient of protein concentration whereas the latter does not.

Since the specific conductance, κ_P, of the protein solution P has been found experimentally to be less than that, κ_B', of the adjusted buffer solution, the electric field is greater in the protein solution than in the buffer and variations of this field thus exist at the descending boundary d. The protein ions in the dilute uppermost layers of the boundary d are therefore in weaker fields than are those in the concentrated layers and thus tend to lag behind, causing the boundary to become diffuse, as is shown in Fig. 4b. In the case of the boundary r rising into the buffer solution $\kappa_{P'} < \kappa_B$ so that the dilute, slowly moving protein ions tend to be overtaken by the faster concentrated ones, with the result that this boundary tends to remain sharp, as is illustrated by Fig. 4a. Due to these field gradients at the boundaries, and in some instances to *p*H gradients also, the distribution through a boundary may be unsymmetrical about the ordinate passing through the maximum of the refractive index gradient curve. This lack of symmetry, shown clearly by the boundary d of Fig. 4b, has been studied by Tiselius and Horsfall.[12] Assuming a proportionality between refractive index and protein concentration it is apparent that in such cases the position of the maximum gradient is not identical with that of the ordinate which divides the area under the curve into two equal parts. The position of the latter ordinate is the better value to use in mobility computations since it is approximately the position the boundary would have if it retained its original sharpness. The precise location of an unsymmetrical boundary requires an integration of the concentration-distance curve. Computations for the cases considered in this paper show that the position of the ordinate dividing the gradient curve into equal parts differs from the true value by at most 1%, whereas the position of the maximum ordinate may be in error by 19%.

It is important in this connection to be clear as to what is meant by the

(11) See MacInnes and Longsworth, *Chem. Rev.*, **11**, 199 (1932).

(12) Tiselius and Horsfall, Jr., *J. Exptl. Med.*, **69**, 83 (1939).

mobility, u, of a protein. It is the distance moved per second in a unit electric field by an average particle in the body of the protein solution. The movement of the particle through this distance corresponds to the transport of $uA[P]$ grams of protein through a plane (to be discussed below) in the body of the protein solution, A being the cross-sectional area of the channel and [P] the concentration of protein in grams per milliliter. If the electric field, F, differs from unity and the transport continues for t seconds the total quantity, p, of protein transported through a reference plane is $p = uA[P]Ft$, from which $u = p/FAt[P]$. Since the field strength, F, in the body of the protein solution is $i/\kappa_P A$ this expression becomes

$$u = p\kappa_P/[P]it \tag{1}$$

in which i is the current. The quantity p is determined analytically in the Hittorf or "transport" method, whereas in the moving boundary method it is determined by following the motion of a boundary between the solution and the buffer. If the passage of electricity causes a boundary to descend from a to d, of Fig. 2, it sweeps through a volume $A(a - d) = V_d$. Thus the amount of protein passing any plane in the unmodified solution is $A(a - d)[P] = V_d[P]$. Equation 1 thus becomes

$$u_d = \frac{A(a - d)[P]}{[P]} \frac{\kappa_P}{it} = \frac{V_d \kappa_P}{it} \tag{2}$$

in which u_d indicates the mobility computed from data on the descending boundary.

The computation of mobilities from data on the rising boundary is, however, complicated by the change of protein concentration at the δ boundary. Assuming, in essential agreement with experiment, that the latter remains stationary, the quantity, p, of protein migrating upward through a plane in the body of the solution on the passage of it coulombs is $(r - a')A[P'] = V_r[P']$ in which [P'] is the concentration of protein in the adjusted solution between the δ and r boundaries. Introducing this value of p into equation 1 we obtain

$$u_r = \frac{[P']}{[P]} \frac{V_r \kappa_P}{it} \tag{3}$$

which differs from equation 2 in that it contains the concentration ratio [P']/[P]. Since it is one of the main conclusions of this paper that, correctly interpreted, the data on rising and descending boundaries yield the same value of the mobility, *i.e.*, $u_d = u_r$, it is important to establish values

of the ratio [P′]/[P]. This requires an investigation, which is given below, of the changes of protein and salt concentrations in the channel of the apparatus during electrolysis.

Strictly, the reference plane from which mobilities are measured should be fixed with respect to the solvent. Since the observed boundary displacements refer to a plane fixed with respect to the apparatus they should be corrected for the displacement the solvent experiences as a result of electrolysis (see reference 11, page 203) otherwise the mobilities found will depend, among other things, upon the nature of the electrode reaction. This computation is greatly facilitated if one electrode chamber is closed since only the volume changes occurring on this side of the protein solution can affect the position of the boundaries. In the experiments reported in this paper the anode chamber was closed and the volume change, ΔV, was equal to

$$\Delta V = f(T_{-}\varphi_{\mathrm{NaAc}} - \varphi_{\mathrm{NaCl}} + V_{\mathrm{AgCl}} - V_{\mathrm{Ag}}) - [\mathrm{P}]V_{\mathrm{d}}\bar{\varphi}_{\mathrm{P}}$$

in which V_{d} is the volume swept through by the descending boundary, for example, on the passage of f Faradays, T_{-} the anion transference number of sodium acetate, φ and V are the apparent molal and molal volumes, respectively, of the materials indicated by the subscripts, and $\bar{\varphi}_{\mathrm{P}}$ is the apparent specific volume of the protein. On the basis of the available data

$$\Delta V = 19.03f - 0.749[\mathrm{P}]V_{\mathrm{d}}$$

A positive value for this volume change represents an expansion of the solution in the closed side and consequently the observed V_{d} is too large by an amount ΔV. The values of V_{d} and V_{r} recorded in Table I have been corrected for this volume change. The corrections for the descending boundary are 2.1, 1.5 and 0.5% at protein concentrations of 0.64, 1.36 and 2.74%, respectively, and are thus by no means negligible. The validity of the correction is difficult to prove in the case of the electrophoresis of proteins but has been amply demonstrated for strong electrolytes.[11]

We have observed repeatedly, within a small experimental error, that (1) the total area of an electrophoretic pattern is independent of the time during the electrolysis at which a pattern may be obtained, *i.e.*, the area is independent of the mode of variation of the refractive index through the boundaries and (2) the total areas of the patterns for the two sides of the channel are identical. These observations are consistent with the fact that the total area of a pattern is given by the integral $\int_{\mathrm{B}}^{\mathrm{P}} (\mathrm{d}n/\mathrm{d}h)\mathrm{d}h$, in which n is the refractive index at the level h in the cell, the limits of integration being the same for both sides of the channel and independent of

the time of electrolysis. Thus the results of planimeter measurements on enlarged images of Figs. 4a and b, in arbitrary units, yielded $A_\epsilon = 18$, $A_d = 354$, $A_\delta = 72$ and $A_r = 302$, from which the sum of the areas for the descending boundaries, $A_\epsilon + A_d$, is 372, whereas the sum, $A_\delta + A_r$, for the rising boundaries has the closely agreeing value of 374.

From the discussion given above it is evident that the partial area A_ϵ of Fig. 4 is due to a gradient of buffer concentration. Since the solution P retains its original composition, in which the buffer electrolyte concentration differs but little from that of solution B, another variation of salt concentration, opposite in sign to that at the ϵ boundary, must exist in the boundary d. Consequently the area A_d must be increased by an amount A_ϵ in order to measure the change of protein concentration at the boundary d. Conductance measurements of the B′ and P′ solutions, to be described later in this paper, indicate that the δ boundary also contains a gradient of buffer salts. Since the same quantity of electrolyte enters the channel from the electrode vessel on one side as leaves the channel on the other side, it follows that the increment of buffer electrolytes in the B′ solution must be compensated by a corresponding decrement in the P′ solution. The concentrations of salts [B] and protein [P] as functions of the height can thus be represented schematically, as is shown in Fig. 5. The increment of buffer salts in the B′ solution is proportional to $A_\epsilon V_d$ while the corresponding and equal decrement in the P′ solution is proportional to $A'_\epsilon V_r$ in which A'_ϵ is that portion of the area A_δ due to the electrolyte gradient in the δ boundary. Therefore $A'_\epsilon = A_\epsilon V_d/V_r$. Moreover, since an electrolyte gradient, equal in magnitude but opposite in sign to that in the δ boundary, must also exist in the boundary r, the area A_r must be increased by an amount $A_\epsilon V_d/V_r$ in order to measure the change of protein concentration at this boundary. The ratio [P′]/[P] of equation 3 thus takes the value $(A_r + A_\epsilon V_d/V_r)/(A_d + A_\epsilon)$ and equation 3 becomes

$$u_r = \frac{V_r\kappa_P}{it} \times \frac{A_r + A_\epsilon V_d/V_r}{A_d + A_\epsilon} \tag{3a}$$

A small error may arise from the use of equation 3a due to the fact that account has not been taken of slight pH changes at the boundaries.

The data necessary for the computation, and the resulting mobilities, at three concentrations of ovalbumin are recorded in Table I. The figures in the second column include data obtained from Fig. 4 and at the other concentrations from similar electrophoretic patterns. The mobilities u_d given in line 13 have been computed with the aid of equation 2 whereas the values u_r in line 12 were obtained from equation 3a. It will be noted

that they agree reasonably closely, as would be expected if our interpretation is correct. Line 11 contains values of u_r^* computed from the relation $\kappa_P V_r/it$, in which the change of protein concentration at the δ boundary, *i.e.*, [P']/[P], has been ignored. The large error which may arise from this procedure may be seen by comparing the figures of lines 11 and 12. The percentage deviations reach a maximum of 37% in our experiments.[13]

It will be observed that the mobilities of ovalbumin, computed as described above and recorded in lines 12 and 13 of Table I, change but slightly with the protein concentration and much of this change disappears if the mobilities are multiplied by the relative viscosity, η (line 10), the product $u_d\eta$ increasing but slightly with the protein concentration. This observation is important since it indicates that mobility measurements may aid in the identification of components in an electrophoretic pattern.

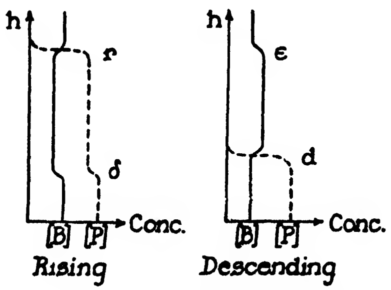

Fig. 5. Schematic diagram of the changes of buffer electrolyte (————) and protein (- - - - - -) concentrations at each of the electrophoretic boundaries.

The Delta and Epsilon Boundaries.—Although the interpretation, given in the foregoing pages, of patterns of the type shown in Fig. 4, appears to be satisfactory, two questions may arise in the mind of the reader. Do gradients of buffer electrolyte really exist in the boundaries? And has the possibility that the δ and ϵ boundaries may be due, in part, to a slowly moving protein constituent been completely eliminated?

(13) This result indicates that mobility measurements involving observations of rising boundaries, without considering the δ effect, may be in error. Thus Landsteiner, Longsworth and van der Scheer (*Science*, **88**, 83 (1938)) used a mean value, $(u_d + u_r^*)/2$, which is about 2.5% higher, for the data of their Table I for example, than the correct value, u_d. It should be noted, however, that this correction does not affect the order of the mobilities, and consequently the conclusions of that paper remain unchanged. In a recent electrophoretic study of hemoglobin Davis and Cohn (This Journal, **61**, 2092 (1939)) observed a mobility increase of 27% on increasing the protein concentration from 0.17 to 1.33%. They computed their results from observations on the rising boundary and it seems probable that most of this apparent mobility variation is due to neglect of the correction for the concentration change at the δ boundary.

To answer the first of these questions the procedure was as follows. After the electrophoresis in which the patterns shown in Fig. 4 were obtained, the contents of each section of the cell were isolated and the specific conductances at 0° were measured. The results, before and after elec-

TABLE I

Electrophoretic Mobilities, u, of Ovalbumin in a 0.1 Normal Sodium Acetate Buffer at pH 3.92 and 0°

1	Protein concn., g. %	0.64	1.36	2.74
2	Conductance, κ_P, at 0°	0.003491	0.003434	0.003326
3	Coulombs, it	143.3	171.7	148.5
4	Volume, V_d, ml.	1.11_4	1.33_9	1.15_3
5	Volume, V_r, ml.	1.24_4	1.60_9	1.58_0
6	A_d, area	169	354	715
7	A_ϵ, area	7	18	36
8	A_r, area	151	302	533[a]
9	A_δ, area	23	72	218
10	Relative viscosity,[b] η, 0°	1.022	1.047	1.095
11	$u_r^* \times 10^5$	3.03	3.22	3.54
12	$u_r \times 10^5$ (Equation 3a)	2.71	2.74	2.63
13	$u_d \times 10^5$ (Equation 2)	2.71	2.68	2.59
14	$u_d\eta$	2.77	2.81	2.84

[a] *By difference; the maximum refractive index gradient of this boundary was too great to be recorded.* [b] *Interpolated from measurements made in this Laboratory.*

TABLE II

Conductance Changes on Electrolysis of a 1.36% Ovalbumin Solution in a 0.1 Normal Sodium Acetate Buffer, at pH 3.92

Section	κ (before)	κ (after)	Diff. in %
Cathode, upper, B	0.00353	0.00336	−5.1
Cathode, lower, P	.00343	.00344	...
Anode, upper, B	.00353	.00353	...
Anode, lower, P	.00343	.00361	+5.3
P′ solution, computed		.00322	

trolysis, are given in Table II. In the lower cathode and upper anode sections of the cell, Fig. 2, no boundaries were present and no significant change of conductance occurred, showing, as would be expected, that the compositions of the solutions B and P are unchanged by the passage of current. In the upper cathode section, however, where the adjusted protein solution P′ has partially replaced the buffer solution B originally present, the specific conductance had decreased 5.1%. A corresponding *increase* in the lower anode section accompanied the partial replacement

of the protein solution P by the adjusted buffer solution B′. The conductance of the solution P′, between the boundaries δ and r, can be readily estimated. The height of the upper cathode section is known, as well as the positions of the r and δ boundaries. In addition data are available, in Table II, on the conductances of the B and P solutions, and of the mixture of these with the P′ solution. These, assuming simple proportionality, are sufficient data for computing the conductance of the latter solution, which is given as "P′ solution, computed" in the table. A change

FIG. 6.

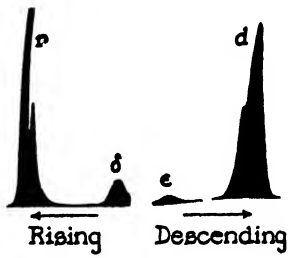

FIG. 7.

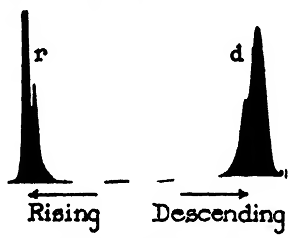

FIG. 6. Electrophoretic patterns of a 1.36% solution of ovalbumin in a 0.1 μ sodium phosphate buffer at *p*H 6.80 and 0°. Scanning exposure made after electrolysis for 6000 seconds at 7.23 volts per cm.

FIG. 7. Same as for Fig. 6 except that the dialyzed ovalbumin solution was diluted in order to eliminate the δ and ε boundaries.

of equivalent conductance from $\kappa_P = 0.00343$ to $\kappa_{P'} = 0.00322$ is therefore indicated at the δ boundary. Since the conductances of these solutions are due mainly to the buffer ions and are roughly proportional to their concentrations, this indicates that there is a decrease of concentration of buffer electrolyte in passing from solution P to solution P′ across the δ boundary in accord with Fig. 5. It is of interest, however, that the ratio $\kappa_{P'}/\kappa_P (= 0.939)$ differs appreciably from that [P′]/[P] (= 0.853) computed from refractive index measurements, showing that the P′ solution is not obtained from the P solution simply by dilution with water as required by the theory of electrophoretic boundaries discussed by Henry and Brittain.[14]

(14) Henry and Brittain, *Trans. Faraday Soc.*, **29**, 798 (1933).

The theory, however, was developed for a ternary ionic system, *i.e.*, protein ions and the anions and cations of a binary buffer salt. At the pH of the experiments and with the buffer solution used (0.1 N NaAc + 0.5 N HAc) it is probable that the effects of the hydrogen ion constituent and of the undissociated acid cannot be neglected.

In this and other laboratories the δ and ϵ boundaries were first thought to be due to slowly migrating protein or carbohydrate constituents.[15] Attempts to isolate such materials were unsuccessful, however. Although the explanation, outlined in this paper, of the boundaries was soon evident[9] the possibility of a small concentration of electrophoretically inert material was not entirely eliminated. The work of Henry and Brittain, mentioned above, suggested a method by which the δ and ϵ boundaries might be suppressed under certain conditions if no inert material were present. In the case of the electrophoresis of ovalbumin in the neutral range, where the contribution of the hydrogen ion conductance to the system is negligible, the conductance ratio $\kappa_{P'}/\kappa_P$ is in fair agreement with the ratio [P′]/[P] determined refractometrically. Thus in the electrophoresis experiment (the patterns for which are shown in Fig. 6) of a 1.36% solution of ovalbumin in a phosphate buffer at 6.80 the conductance, $\kappa_{P'}/\kappa_P$, and protein concentration, [P′]/[P], ratios were found to have nearly the same value, *i.e.*, 0.935 and 0.923, respectively. Under these conditions the concentrations of the constituents of the P′ solution are such as would result from dilution of the P solution with water. Thus in this case if the boundaries were initially formed between the buffer and the solution P′, obtained by diluting solution P by the factor 1/0.935, there should be no δ and ϵ boundaries left behind when the protein boundaries migrate away from their original positions. This idea was tested experimentally with the result shown in Fig. 7. It will be seen that the δ and ϵ boundaries, quite evident in Fig. 6, have been eliminated. This would appear to be conclusive evidence of the nature of the δ and ϵ boundaries, as outlined above, and of the absence of inert constituents in the sample of ovalbumin.

SUMMARY

Electrophoretic studies of ovalbumin, at 0° and at pH 3.92, have been made by the moving boundary method. The research was undertaken in order to establish the conditions under which accurate values of protein mobilities may be found from moving boundary data. This involved investigation of the nature of the δ and ϵ boundaries, and of the concentra-

(15) Tiselius, *Biochem. J.*, **31**, 1464 (1937).

tion distributions in the rising and descending protein boundaries. The results indicate that, although in general more diffuse, the descending boundaries yield correct values of the mobility. The rising boundaries may give results that are greatly in error unless additional data are available from which corrections may be made, in which case the two types of boundary yield the same value of the mobility. Contrary to some recent conclusions, the mobilities have been found to vary but slightly with the protein concentration, if a small viscosity correction is made, within the composition limits studied. *There is evidence that gradients of buffer concentration exist, in general, in all the boundaries. The correctness of the interpretation has been indicated by showing that, in certain cases, the* δ and ϵ boundaries may be suppressed.

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SEMIQUINONES OF OXAZINES, THIAZINES AND SELENAZINES

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1. After it had been shown[1] that thionine and methylene blue yield free semiquinone radicals on partial reduction, a more systematic study of the dyestuffs of this class was suggestive. It will be shown in this paper that its various representatives vary greatly with respect to the magnitude of the formation constant and the optical properties of the semiquinone. The investigations were extended over suitable representatives not only of thiazines, but also oxazines and selenazines. The better known dyestuffs with two auxochromic amino groups such as methylene blue were compared with those containing only one, either an amino or a hydroxy group. The experiments will show that all of these compounds easily form semiquinones, under similar conditions as does thionine, namely, in sufficiently acid solution. In part the separation of the two steps of oxidation is very distinct even in weakly acid solutions, especially for 3-hydroxythiazine and 3-hydroxyoxazine. In these two compounds, on increasing the acidity, it is easy to reach the value 10^{+6} for the semiquinone formation constant, by far the largest constant of this type encountered as yet. All these semiquinones show a characteristic absorption spectrum in the visible range of wave lengths. There are two types of absorption spectra: either there is one rather intense and sharp band; or there is such a complicated series of bands as recently described for thionine and methylene blue. It is not yet possible to correlate the chemical structure with the type of absorption spectrum. In every case the spectrum of the semiquinone is quite different from that of the quinonoid form of the dye. The high stability of those radicals, often largely exceeding that of the thionine radical under comparable conditions, was rather unexpected from the theoretical point of view as tentatively adopted in the previous paper, and will be commented on in the discussion.

2. *Material.*—The nomenclature of these dyestuffs is rather cumbersome. Bernthsen's nomenclature as applied in his classical papers is not systematic enough to cover derivatives in addition to those investigated by him. The

(1) L. Michaelis, M. P. Schubert and S. Granick, THIS JOURNAL, 62, 204 (1940).

TABLE I

3-Aminothiazine

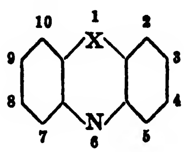
Titrations in 1 × 10^{-4} M Solution; 50 Cc. Increase in Volume during Titration, 2 Cc. Approx.

Solvent	Manner of titration	pH measured at the H_2 electrode after titration	E_m Potential at 50% oxidation, referred to the standard H_2 electrode	E_i Index potential Left	E_i Index potential Right	Comments
Phosphate + NaOH	Reduced with Pd-H_2 Titrated with $K_3Fe(CN)_6$	11.25	+.013	15.5	15.7	
Veronal buffer	Same	8.66	+.152	?	16.0	In the first half of the titration potentials have little poise. E_m is probably reliable
Phosphate	Same	7.16	+.205	?	?	Poise not sufficient for a reliable E_i. E_m is approximately correct
Phosphate	Reductively titrated with leuco-rosinduline GG	6.73	+.219	15.3	15.4	Unobjectionable in every respect
Phosphate	Reduced with Pd-H_2, titrated with $K_3Fe(CN)_6$ dissolved in same buffer	6.15	+.236	16.0	15.5	Unobjectionable in every respect
Acetate	Reductively titrated with leuco-rosinduline GG	4.62	+.293	15.4	15.6	Unobjectionable in every respect
Citrate	Reduced with Pd-H_2, titrated with $K_2Cr_2O_7$, dissolved in pure H_2O	1.91	+.452	15.4	15.3	Unobjectionable in every respect
Citrate	Same	1.02	+.506	15.9	15.8	Unobjectionable in every respect
0.990 N HCl	Same	0.07	+.558	18.5	16.4	Slight drifts, rendering E_i unreliable
1.11 N H_2SO_4	Reduced with Pd-H_2, titrated with ferric ammonium sulfate dissolved in same acid	0.29	+.548	15.6	15.3	Establishment of potential takes about one minute, but final values appear to be reliable
3.58 N H_2SO_4	Reductively titrated with chromous sulfate in same acid	(−0.22?)	(+.573)	16.3	16.6	

TABLE I—*Concluded*

Solvent	Manner of titration	pH measured at the H_2 electrode after titration	E_m Potential at 50% oxidation, referred to the standard H_2 electrode	E_i Index potential		Comments
				Left	Right	
6.64 N H_2SO_4	Reductively titrated with titanous sulfate		(+.583)	20.0	19.2	
8.88 N H_2SO_4	Same		(+.581)	30.5	30.2	
11.1 N H_2SO_4	Same		(+.569)	54.5	54.0	Similar experiment with chromous sulfate gave less sharp end-point due to overlapping, but otherwise the same
15.3 N H_2SO_4	Same			94 approx.		End-point overlapping with Ti, yet recognizable

nomenclature of the leuco compounds is always quite easy, so we recommend using always the name of the leuco dye. The prefix "pheno" in phenothiazine, phenoxazine or phenoselenazine may be omitted. Whenever it is necessary to distinguish the leuco compounds from their oxidation products, we add the prefix r-, s- or t-, to distinguish the reduced form, the semi-oxidized or semiquinone form, and the totally oxidized or quinonoid form. For instance, thionine then is t-3,9-diaminothiazine, and when no misunderstanding arises the t- is omitted. The numbering of the skeleton as shown is the customary one. "X" stands for O in oxazine, for S in thiazine, and for Se in selenazine



The substances were all prepared according to methods already described in the literature and only a few notes need be added where modifications were made.

Phenoxazine.[2]—Best results were obtained when the *o*-aminophenol and its hydrochloride were both recrystallized until almost white before proceeding with the condensation. Eighteen grams of *o*-aminophenol and 22 g. of its hydrochloride are heated

(2) F. Kehrmann and A. A. Neil, *Ber.*, 47, 3107 (1914).

under carbon dioxide at 240° for thirty minutes. The reaction mixture is extracted with 1200 cc. of hot benzene and the residue after evaporating the benzene is dissolved in 250 cc. of hot alcohol. To the hot alcoholic solution are added 100 cc. of water and 100 cc. of concentrated hydrochloric acid and the solution is filtered hot from a tarry deposit.[3] Addition of 2 liters of water to the filtrate deposits the crude phenoxazine. This is dried, distilled at 215° and 4 mm. and the product recrystallized from 100 cc. of hot alcohol by addition of 200 cc. of water. Thirteen grams is obtained with a m.p. of 151–152°.

Phenothiazine was prepared by the method of Sakom.[4] After distillation at 190° and 3 mm. it is recrystallized from benzene with petroleum ether.

Phenoselenazine[5] was distilled at 200° and 1–2 mm. and recrystallized from alcohol and then from benzene. The selenium monochloride required was prepared by the method of Lenher and Kao.[6]

t-3-Hydroxythiazine was prepared by the method of Pummerer and Gassner[7] though the crude product reported by these authors to have a melting point of 162–163° was further purified. About 4 g. of the crude product was first extracted with 100 cc. of hot alcohol and the crystalline product obtained by chilling this extract was further recrystallized from 1500 cc. of boiling water. The crystalline product, 0.7 g., had a melting point of 161°.

t-3-Hydroxyoxazine was prepared by a similar method from phenoxazine. After two recrystallizations from alcohol, using about 50 cc. per g., a crystalline product is obtained which decomposes at 214°.

t-3-Aminophenothiazine[8] and *t-3-aminophenoselenazine*[4] were used as their simple hydrochlorides.

t-3-Amino-9-hydroxythiazine can be obtained easily although in small yield by saturating with hydrogen sulfide a solution of 20 g. of *p*-aminophenol in 1 liter of water containing 20 cc. of concentrated hydrochloric acid. A solution of 100 g. of $FeCl_3 \cdot 6H_2O$ in 150 cc. of water is added, the mixture is aerated and another equal amount of ferric chloride is added. The crude precipitate is extracted with 1 liter of boiling water containing 50 cc. of concentrated hydrochloric acid and the crystalline product resulting on cooling is recrystallized from 300 cc. of water containing 15 cc. of concentrated hydrochloric acid. The crystalline product is the hydrochloride of Bernthsen's thionolin[9] and contains one mole of water.

t-3,9-Diaminophenoxazine hydrochloride is obtained in brilliant crystals by the method described by Kehrmann and Saages[10] and t-3,9-diaminophenoselenazine hydrochloride as described by Cornelius.[5]

The selenium analog of methylene blue can be made simply as described by Karrer[11]

(3) F. Kehrmann, *Ann.*, **322**, 9 (1902).
(4) D. Sakom, *J. prakt. Chem.*, **89**, 11 (1914).
(5) W. Cornelius, *ibid.*, **88**, 395 (1913).
(6) V. Lenher and C. H. Kao, This Journal, **47**, 772 (1925).
(7) R. Pummerer and S. Gassner, *Ber.*, **46**, 2324 (1913).
(8) A. Bernthsen, *Ann.*, **230**, 100 (1885).
(9) A. Bernthsen, *ibid.*, **230**, 201 (1885).
(10) F. Kehrmann and A. Saages, *Ber.*, **36**, 475 (1903).
(11) P. Karrer, *ibid.*, **51**, 190 (1918).

although the product obtained by crystallization from weak hydrobromic acid and dried in vacuum at room temperature contains an extra half mole of hydrogen bromide. Calcd. for $C_{16}H_{18}N_3SeBr \cdot H_2O \cdot \frac{1}{2}HBr$: N, 8.94; Br, 25.52. Found: N, 9.04; Br, 25.85.

t-3,9-Dihydroxyphenothiazine could not be prepared in any pure form. The method of De Eds and Eddy[12] gave a product of little promise. The original method of Bernthsen gives poorly crystalline products and uncertain reproducibility. In one trial only a well-crystallized product was obtained which only roughly approximated in composition the hydrochloride. Calcd. for $C_{12}H_8O_2NSCl$: N, 5.28; S, 12.05; Cl, 13.35. Found: N, 5.57; S, 10.88; Cl, 11.68. Since none of our preparations showed steady potentials in potentiometric titrations, this substance was not included in the following studies. It is mentioned only with reference to the paper by De Eds and Eddy.

t-3,9-bis-Phenylaminothiazine Bromide.—Five grams of the perbromide of thiazine prepared as described by Kehrmann[13] is suspended in 15 cc. of methanol, 10 cc. of aniline is added and the mixture thoroughly ground up. The product crystallizes out directly. Twenty-five cc. of ether is added, the product is filtered off and washed with ether. It is recrystallized once or twice from 800 cc. of hot alcohol from which it separates as large shining crystals on chilling; yield 1.0 g.; no m. p. up to 298°. Calcd. for $C_{24}H_{18}N_3SBr$: N, 9.13; S, 6.95; Br, 17.36. Found: N, 9.05; S, 6.26; Br, 17.88. Of this compound only the absorption spectrum of the semiquinone is shown in Fig. 8.

3. *Methods of Potentiometric Titration.*—Titrations in strongly acid solutions were performed with liquid junctions such as described before, in the form of agar bridges previously adapted to the acid solution so as to establish non-drifting liquid junction potentials. Titrations were made in part reductively, in part oxidatively. For reductive titration, titanous chloride dissolved in the corresponding acid was used. Or, chromous sulfate was used as follows. A suitable amount of chromium metal powder was put in deaerated sulfuric acid of the desired concentration. From one to eleven *N* sulfuric acid may be used; in higher concentrations the metal dissolves as a chromic instead of a chromous salt. The dissolving process is speeded up by heating in a stream of nitrogen. The solution of chromous sulfate in sulfuric acid is directly transferred by nitrogen-pressure to the buret. Whenever the potential range was suitable this reductant was very satisfactory. However, contrary to expectation, the potential range in sulfuric acid of higher concentration is less negative than that of titanous sulfate. Oxidative titrations also were performed. They often permitted covering even a larger potential range. They were carried out by reducing the dye solution with a small drop of 1% colloidal palladium and hydrogen gas, thoroughly displacing the hydrogen by nitrogen, and titrating with a deaerated solution of potassium bichromate dissolved in the same acid.

Titrations in less acid solutions, within the *p*H range of the customary buffers, were performed either reductively with leuco-rosindulin GG, or, oxidatively, after reducing with palladium and hydrogen, with potassium ferricyanide, and sometimes with ferric ammonium sulfate.

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(12) F. De Eds and C. W. Eddy, This Journal, **60**, 1446 (1938).

(13) F. Kehrmann, *Ber.*, **49**, 53 (1916).

4. *Results of Potentiometric Titrations*

(*A*) *Thiazine Derivatives.* (*1*) *3-Amino-thiazine.*—The results are shown in Table I and Fig. 1.

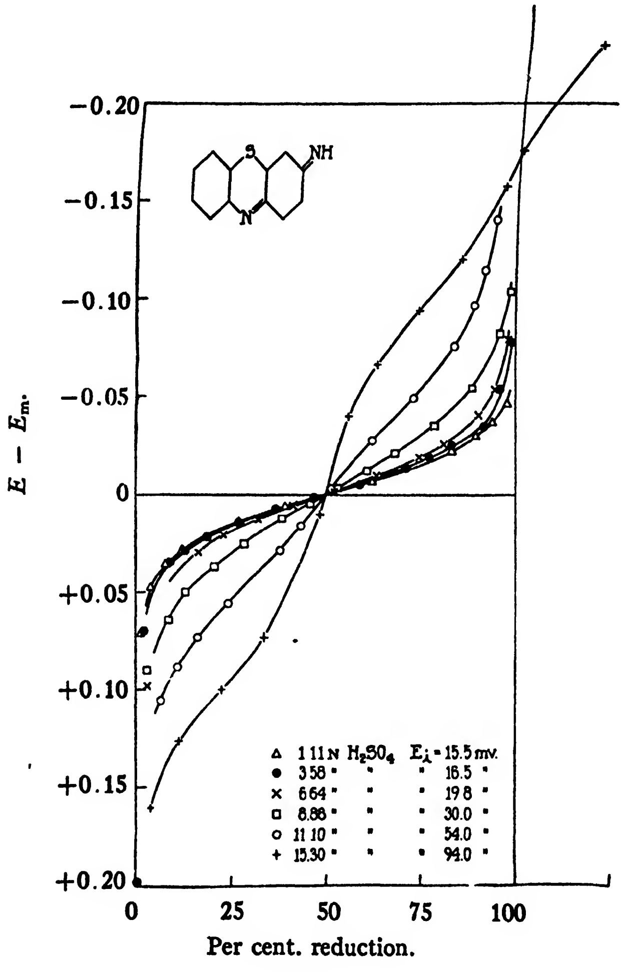


Fig. 1

(*2*) *3-Hydroxythiazine.*—This dyestuff is of particular interest because a distinct step formation becomes manifest already in rather weakly acid solutions, well definable in terms of *p*H. This fact itself would not be so exceptional since it has been observed before for other cationic dyestuffs, as for pyocyanine and related compounds. However, on increasing the acidity, for the latter dyestuffs very soon there arises the condition that the potential range of the lower step overlaps with, or even far exceeds, the

otential of hydrogen for the same acidity. This overvoltage renders a full titration over both steps impossible. For 3-hydroxythiazine there prevails

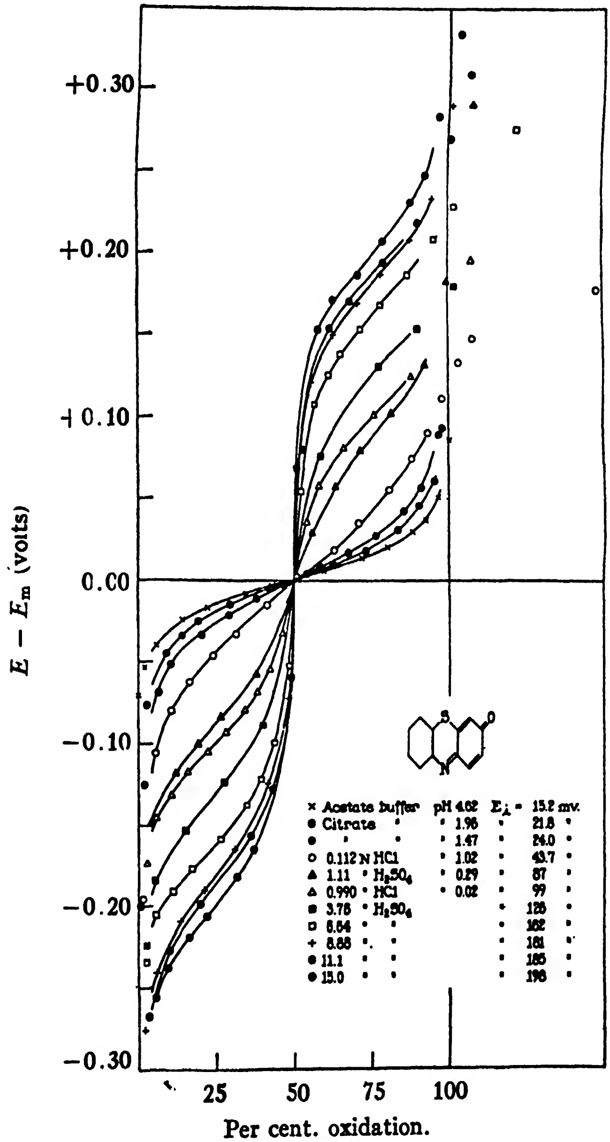


Fig. 2

the rare opportunity that both steps can be titrated over an enormous range of acidity without any overlapping with the hydrogen potential occurring. The results are shown in the same fashion as with 3-aminothiazine,

TABLE II

3-Hydroxythiazine

Concentration always 1×10^{-4} M, Except for the Experiment at pH 12.59

Solvent	Manner of titration	pH	E_m	E_i Left	E_i Right	E_2-E_1	Comments
Sec. phosphate + NaOH	Reductive titration with leuco-rosinduline GG	12.59	−0.103 approx.				(See footnote[a])
Phosphate	Same	6.77	+ .159	15.2	15.0	−0.12	
Acetate	Same	4.62	+ .288	14.9	15.0	− .12	
Acetate	Same (another sample of the dye)	4.62	+ .287	15.3	15.4	− .12	
Citrate	Reduced with Pd-H_2, titrated with $K_2Cr_2O_7$	1.96	+ .449	19.4	19.5	− .022	
Citrate	Same	1.47	+ .477	26.0	25.8	+ .024	
0.112 N HCl	Dye dissolved in 0.5 cc. of alcohol; added 50 cc. of 0.112 N HCl. *Reduced with Pd-H_2, titrated with ferric ammonium sulfate*	+1.02	+ .506	43.6	43.8	+ .081	End-point overlaps somewhat with Fe-potential
1.11 N H_2SO_4	Same procedure, with 1.11 N H_2SO_4, and titrated with $K_2Cr_2O_7$	+0.29	+ .543	87		+ .174	
0.990 N HCl	Dissolved similarly in 0.990 N HCl, titrated with titanous chloride dissolved in HCl of same concn.	+0.02	+ .555	99	99	+ .198	
3.76 N H_2SO_4	Reduced with Pd-H_2, titrated with $K_2Cr_2O_7$		+ .553			.252	
3.76 N H_2SO_4	Reductive titration with Ti^{+++}		+ .553			.242	
6.64 N H_2SO_4	Same					.324	Overlapping with Ti-potential at the end of titration
8.88 N H_2SO_4	Reduced with Pd + H_2, titrated with $K_2Cr_2O_7$					.363	
11.1 N H_2SO_4	Same					.371	
15.0 N H_2SO_4	Same					.397	

[a] At this pH the dye was very little soluble. The solution was filtered, the poise of the potential not good enough to yield reliable values for E_i.

in Table II, and Figs. 2 and 3. Figure 2 corresponds to Fig. 1. Since the separation of the steps spreads into the well measurable pH range, not only E_m, but also E_1 and E_2 can be plotted against pH (Fig. 3) in the same way as has been done previously for pyocyanine and many other dyestuffs. It can be seen that at pH around 1 the uncertainty due to the liquid junction potentials begins. The observed points no longer fit the theoretical curves. For instance, the experimental points for E_1 at pH values < 1 apparently indicate that E_1 decreases a little with decreasing pH. This is impossible. There is obviously something wrong with the absolute potential values. This uncertainty sets a limit to extending the diagram,

TABLE III

3-Hydroxy-9-Aminothiazine

Solvent	Manner of titration	pH	E_m	E_1 Left	E_1 Right	Comments
Phosphate	Reductively with leuco-rosinduline GG	7 11	−0.220 approx.	..	..	Satd. solution, < 10^{-4} M
Citrate	Same	2.92	+ .072	16.0	16.5	Concentration 6×10^{-5} M
4.52 N H_2SO_4	Reduced with Pd + H_2, titrated with $K_2Cr_2O_7$	.	(+ .263)	24	24	Concentration 5×10^{-5} M
7.60 N H_2SO_4	Reduced with Ti^{+++}	.	(+ .261)	48	52	Concentration approximately 1×10^{-4} M
15.0 N H_2SO_4	Reduced with Pd + H_2, titrated with $K_2Cr_2O_7$	..	(+ .245)	110		

Fig. 3, further to the left, so the abundant experimental material can only be demonstrated in the way of Fig. 2. One might have attempted to extend the graph, Fig. 3, into the region of negative pH values by expressing the pH of the stronger sulfuric acid concentrations according to Hammett's[14] extended acidity scale. This problem is too important to discuss in a perfunctory way. It is rather to be considered as a special topic for a later investigation and will not be entered upon now.

(*3*) *3-Hydroxy-9-aminothiazine.*—This dyestuff is too little soluble within the ordinary pH range to allow really accurate titration experiments. Two titration experiments are shown, one at pH 7.11, the other at pH 2.92 (Table III), to show approximately the normal potentials in comparison with the other compounds. In more acid solutions the solubility is somewhat better so that the desired concentration of approximately 1×10^{-4} M

(14) Hammett, *Chem. Rev.*, **16**, 67 (1935).

can be reached. Figure 4 shows the family of titration curves for varied concentration of sulfuric acid.

(*B*) *Oxazine Derivatives.* (*5*) *3,9-Diamino-oxazine.*—3,9-Diamino-oxazine is quite analogous to thionine (Lauth's violet). Its aqueous solution shows a gorgeous red fluorescence. Its normal potential E_m can be read

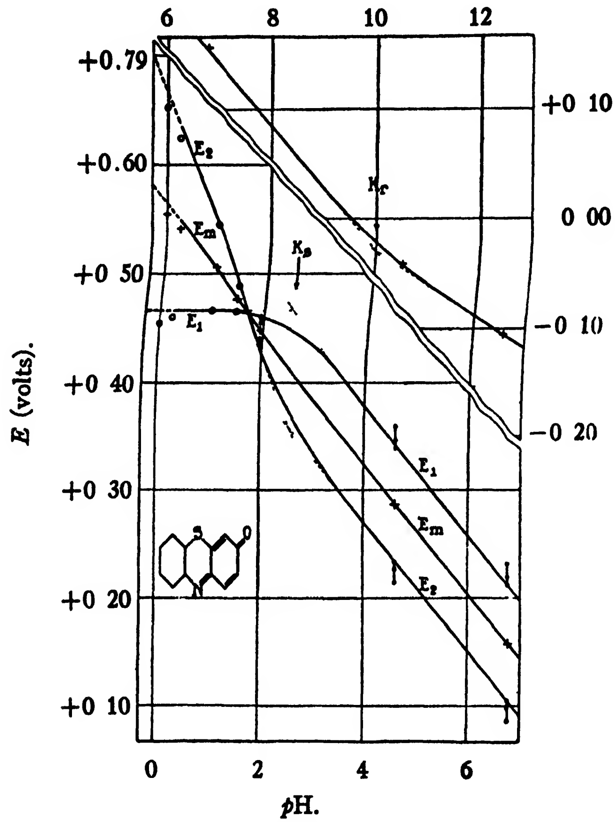


Fig. 3

from Fig. 6, and the separation into two steps is distinct in acid solution as shown in Table IV. The separation is smaller than that for thionine, compared in solutions of the same acidity. The index potential is 22 millivolts in 20.8 *N* sulfuric acid; it was 79 millivolts for thionine in sulfuric acid of nearly the same concentration (20.4 *N*).

(*6*) *3-Hydroxy-oxazine.*—The results are described in Table V and Fig. 5. The separation of the two steps is quite similar to that in 3-hydroxythiazine.

(*C*) *Selenazine Derivatives.* (*7*) *3,9-Diaminoselenazine.*—The experi-

ments tabulated in Table VI show a great similarity in every respect to thionine.

(8) *3,9-Di-(dimethylamino)-selenazine* (Selenium-methylene blue).—The semiquinone formation for a given acidity is much smaller than in the pre-

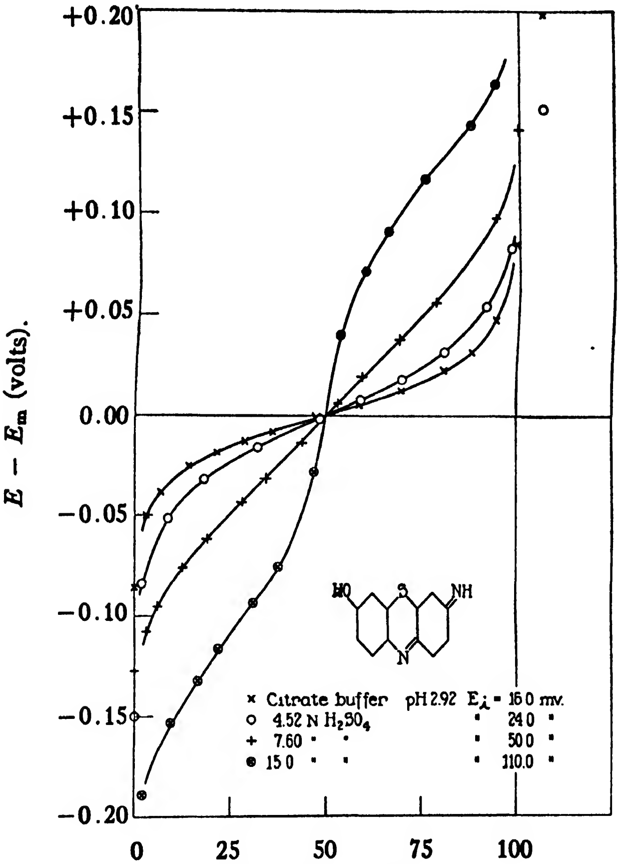


FIG. 4

ceding dyestuff. This difference is just the same as that between methylene blue and thionine.

Figure 6 is a plot of the mean normal potential, E_m, against *p*H for several of the dyestuffs investigated in this paper, and some others for comparison.

5. *The Optical Properties of the Dyes.*—Comparing the spectrum of the various radicals of the thiazines, oxazines and selenazines, two general types of absorption spectra may be distinguished, and no, or at least no

distinct, transitional types have been observed. One type is that described for the semiquinones of thionine and methylene blue before, with a series of bands in the green region of the spectrum exhibiting a fine structure not often encountered in organic dyestuffs and, in addition, a broader band

TABLE IV

3,9-Diamino-Oxazine

Solvent	Manner of titration	*p*H	E_m	E_i Left	E_i Right	Concn.
Phosphate	Reductive titration with leuco-rosinduline GG	6.79	−0.003	15.3	15.5	1×10^{-4}
Phosphate	Same	6.16	+ .018			1×10^{-4}
Citrate	Same	3.73	+ .169	14.9	14.6	1×10^{-4}
Citrate	Reduced with Pd + H_2; titrated with $K_2Cr_2O_7$	2.32	+ .294	14.3	14.3	1×10^{-4}
15.6 *N* H_2SO_4	Reduced with Cr^{++}			15.5	15.6	2×10^{-4}
20.8 *N* H_2SO_4	Same			23	22	2×10^{-4}

TABLE V

3-Hydroxyoxazine

Solvent	Procedure	*p*H	E_m	E_i Left	E_i Right	$E_2 - E_1$
Acetate buffer	Titrated with leuco-rosinduline GG	4.62	−0.0284	15.3	15.1	
Citrate	Reduced with Pd + H_2; titrated with $K_2Cr_2O_7$	2.02	+ .1304	15.1	15.3	
4.52 *N* H_2SO_4	Same					0.206
Same	Same					.205
7.60 *N* H_2SO_4	Same					.262
16.6 *N* H_2SO_4	Same					.301

within the far blue without finer structure. The other type shows only one intense and rather sharp band.

Figure 9 shows the absorption of the quinonoid forms of some of the dyestuffs. This figure is to show especially that thionine (in the form of its simply charged cation as existing in mildly acid solution), does not obey Beer's law.[15] The same to a lesser degree holds for t-diamino-oxazine. In addition it is shown in two examples how strongly the absorption is diminished by removing one of the two symmetrically located amino groups,

(15) *Cf.* quotation (1), footnote on page 210.

or replacing one of them by OH, making the molecule unsymmetric with respect to the two "auxochromic" groups. The absorption spectra plotted

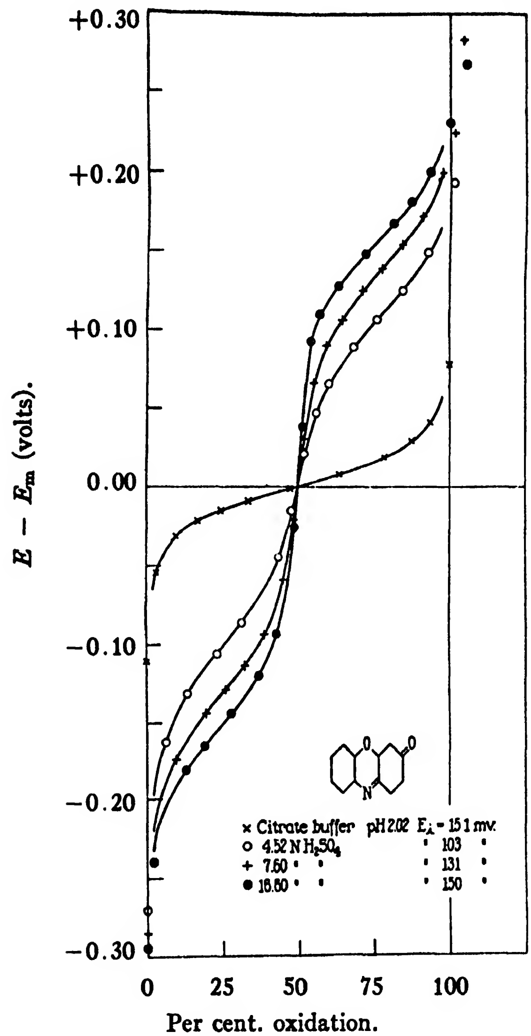


FIG. 5

in Fig. 9 hold for those states of ionization of the quinonoid form as indicated in the formulas of the legend. It is noteworthy that the great difference in the molar extinction coefficient according to the nature of the auxochromic groups holds only for the quinonoid forms, and not for the semiquinones (Fig. 7). In the quinonoid forms, the absorption is strongest if there are two amino groups in position 3 and 9 so as to establish an *equivalent*

benzene-quinone resonance. Absorption is much weaker when there is only one auxochromic group, or two such of opposite character (one NH_2 and one OH), whereby the resonance is decreased. No such distinction prevails among the semiquinones.

TABLE VI

3,9-Diaminoselenazine

Solvent	Manner of titration	*p*H	E_m	E_i	
				Left	Right
Phosphate	Reduced with Pd + H_2; titrated with $K_3Fe(CN)_6$	6.76	−0.107	14.8	15.0
Acetate	Titrated with leuco-rosinduline GG	4.62	− .097	15.3	15.5
Citrate	Same	3.09	+ .319	15.4	15.5
16.6 *N* H_2SO_4	Titrated with Ti^{+++}		+ .615	22.3	22.1
18.9 *N* H_2SO_4	Titrated with Ti^{+++}		+ .626	46.5	46.5

TABLE VII

3,9-Di-(Dimethylamino)-Selenazine

Solvent	Manner of titration	*p*H	E_m	E_i	
				Left	Right
Phosphate	Titrated with leuco-rosinduline GG	6.73	+0.049	17	17
Acetate	Reduced with Pd + H_2; titrated with $K_3Fe(CN)_6$	4.62	+ .159	14 (approx.)	
Citrate	Titrated with leuco-rosinduline GG	1.94	+ .382	15.8	16
23.0 *N* H_2SO_4	Titrated with Ti^{+++}		+ .538	27	28

DISCUSSION

On attempting to explain a relatively high stability of a free radical, or any other molecular species, which according to classical structural formulas should be expected to be unstable, the principle of resonance may account for an increase of stability beyond the expected extent. This is, at the present stage of the theory, a rather vague qualitative statement since it is practically impossible at the present time to apply this principle quantitatively for compounds of such complicated structure as we have to deal with. The case of triphenylmethyl is easier for quantum mechanical treatment and was successfully dealt with by Pauling and Wheland,[16] and by Hückel,[17]

(16) L. Pauling and Wheland, *J. Chem. Phys.*, **1**, 362 (1933).
(17) E. Hückel, *Z. Physik*, **83**, 632 (1933).

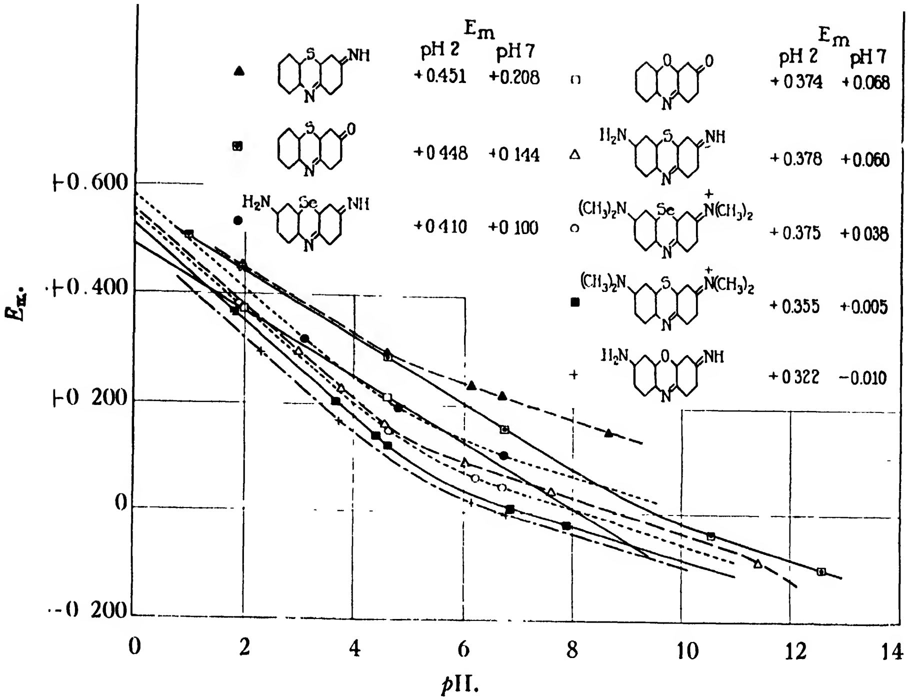


FIG. 6. Normal potentials, E_m, plotted against pH.

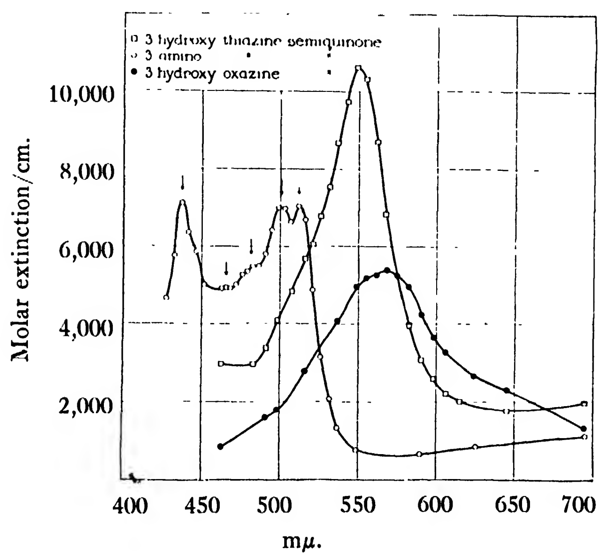


FIG. 7. The dyes were dissolved in 15 N sulfuric acid. The concentration of the S-form was 1.5×10^{-4} M, and a 1-cm. cell was used in the König-Martins spectrophotometer. The S-forms were prepared by mixing equal parts of the fully oxidized, deaerated form, with the fully reduced form (reduced with Pd + H_2, then hydrogen displaced by nitrogen). The S-forms are scarcely autoxidizable under these conditions.

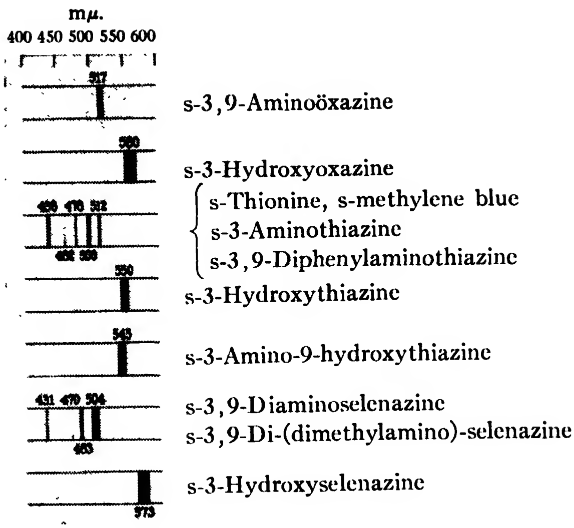


FIG. 8. The S-forms were prepared by dissolving the oxidized form of the dye in suitable concentrations of sulfuric acid and partially reducing with titanous sulfate. The positions of the bands were located with a hand spectroscope. The 512 mμ band of s-thionine and s-methylene blue is more diffuse than represented in the figure.

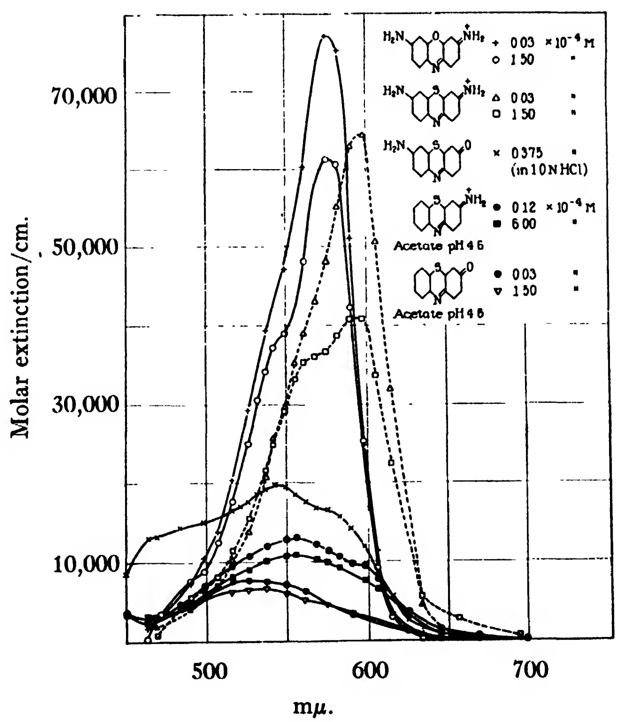
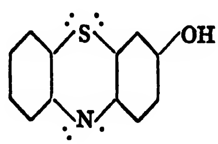
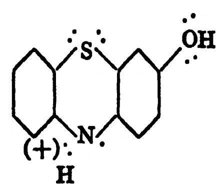


FIG. 9

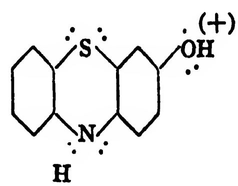
using two different methods of approximation. It will be much more difficult to use these methods for the compounds described in the present paper. Some qualitative considerations have to suffice for the time being. In the preceding paper it was suggested that the resonance structure of thionine is analogous to that of Wurster's dyes which because of their structural simplicity were used as models. However, in going over the compounds investigated in this paper, we encounter very stable radicals for which no immediate analogy with Wurster's dyes is obvious.



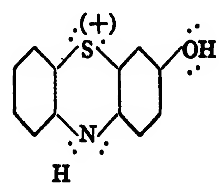
I



II



III



IV

Experience as obtained from the examples of this paper shows that a radical such as I is stabilized by acidity, by attaching a proton, as in II. The latter structure is in resonance with III. This, however, is no longer an *equivalent* resonance. Although it may be generally true that equivalent resonance is a highly stabilizing factor there is no reason why a non-equivalent resonance sometimes should not exert a highly stabilizing effect, too. If so, there is no longer any reason to restrict the limiting structures to the types previously shown. Another considerable contribution to stability may be attributed to a resonance between a pair of limiting structures, one of which is II, the other IV, with the odd electron in II at the N, in IV at the S. With this type of resonance, no side chain in phenothiazine would be necessary at all to establish a fairly well stable radical.

The logical continuation of these ideas would lead to an investigation of radicals derived by partial oxidation from unsubstituted phenothiazine, phenoxazine or phenoselenazine. Experiments along these lines will be the subject of a later publication.

SUMMARY

The formation of free semiquinone radicals in a number of oxazine, thiazine and selenazine dyestuffs is investigated. All of these dyes easily form semiquinones in sufficiently acid solutions. The semiquinone formation constants for some of these dyestuffs are the largest encountered as yet, *e. g.*, amounting to 10^{+6} for 3-hydroxythiazine in 15 N sulfuric acid. Two types of absorption spectra for the semiquinones of these classes of dyestuffs are described, one consisting of a single rather sharp band, the other consisting of a complicated band system. High stability of these radicals is encountered not only when the resonance is an equivalent one, but also under certain conditions, when the resonance is not quite equivalent and a complete analogy to the structure of Wurster's dyes is missing.

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EXPERIMENTS ON CULTIVATION OF VIRUS OF INFECTIOUS AVIAN ENCEPHALOMYELITIS*

BY I. J. KLIGLER AND P. K. OLITSKY

(*From the Laboratories of The Rockefeller Institute for Medical Research*)

In connection with investigations on the virus of infectious avian encephalomyelitis (A.E.),[1] we sought to enhance antigen quantitatively for the preparation of immunizing vaccines, by cultivating the virus in developing chick embryos and in their tissues *in vitro*. The results relating to epidemiology and to additional properties of this newly discovered virus were considered of sufficient importance to be offered in the present paper.

Materials and Methods. The strain of virus employed was that kindly sent to us by Dr. Van Roekel and which was described by him[1] as well as by Olitsky.[1] The procedures followed closely those which had been employed by the latter. Ten percent infected-chick-brain suspension in broth was used to initiate the various cultures. Tests for virus, except as noted otherwise, were made by intracerebral inoculation of 0.05 to 0.1 cc of tenfold dilutions of the material to be examined into 2- or 3-weeks-old Rhode Island, or New Hampshire, Red chicks.

Fertile eggs of White Leghorn chickens were available. It was shown that of 11 normal, 2-week-old birds of this breed given 0.05 cc of 10^{-1} dilution of active brain intracerebrally, all developed characteristic encephalomyelitis after 11 to 16 days. Hence the Leghorn chicks are susceptible to the A.E. virus.

Cultivation Experiments. 1. *In Developing Chick Embryos.* In view of the fact that the virus is active only in avian hosts and that it has been reported[2] that the disease can be transmitted by way of the egg, the first attempts at cultivation were made in embryonated eggs as the most promising approach to the problem. Since the virus was shown to be active in nervous tissue, it was thought desirable to employ embryos of eggs of 5

* We express our debt to Mr. P. Haselbauer for his invaluable aid.

[1] Olitsky, P. K., *J. Exp. Med.*, 1939, **70**, 565; Olitsky, P. K., and Bauer, J. H., PROC. SOC EXP. BIOL. AND MED., 1939, **42**, 634. For earlier descriptions, see: Jones, E. E., *J. Exp. Med.*, 1934, **59**, 781, and Van Roekel, H., Bullis, K. L., and Clarke, M. K., *J. Am. Vet. Med. Assn.*, 1938, N.S. **46**, 372.

[2] Van Roekel, H., Bullis, K. L., and Clarke, M. K., *Vet. Med.*, 1939, **34**, No. 12.

or 6 days' incubation, which had been used successfully for the cultivation of rabies-virus.[3]

Parallel cultures were set up, employing 1,000 chick cerebral-test-doses of A.E. virus, one group being inoculated by way of the yolk-sac, after the manner of Cox;[4] another, through an opening over the embryo, directly into the allantois. The eggs were then incubated at 36.5°C for 6–10 days, when their contents were removed and passage made from the allantoic membrane, yolk-sac, embryonic tissue, and brain to new series of eggs. The different tissues of the 2d and 4th passages were ground in broth and decimal dilutions of 10^{-2} to 10^{-5} or 10^{-6} were injected intracerebrally into chicks. Five series of cultures were made and tested in 175 chicks, and in no instance could viral multiplication be discerned. In 2 instances, the brains of chicks hatched from such inoculated embryos were passaged in young chicks with negative results. Pathological examination of the CNS of embryos 18–20 days old (*i.e.*, 12–14 days after inoculation), or of hatched chicks failed to reveal the characteristic lesions of encephalomyelitis.

In addition, tests were made on the survival of virus injected into young and older embryos. In the former, 21 eggs with 5- to 7-day-old embryos, and in the latter, 11 eggs with 11- to 13-day-old ones were used; 0.1 cc of 10^{-1} dilution of virus (active in 10^{-5} dilution) was inoculated into the eggs through the allantois in the region of the embryo. The presence of virus in the embryos was demonstrated by pooling 2 or 3 inoculated embryos 1 hour later and injecting decimal dilutions of the suspension into chicks. After incubation at 37°C for varying periods, the embryonic brain and the other tissues were again examined for virus-content. In the series of young embryos, virus was recovered in dilutions up to 10^{-3} after 24, 48, and 72 hours, but not at all after 5 or 9 days; in the older ones, virus was recovered in a dilution of 10^{-4} after 48 hours, but not after 8 and 11 days. Pathological examination of the brain and cord of these embryos 11 days after inoculation failed to show any characteristic lesions.

Finally, 21 of the birds which had hatched out of inoculated eggs or had received intracerebral inoculation of the mentioned embryonic material, and failed to show signs of the malady for at least 40 days, were then subjected to an intracerebral test for immunity. All came down with characteristic A.E. virus infection, thus indicating the absence of active multiplying virus in the chicks and in the materials used in an amount sufficient to induce immunity.[1] At the time of the test, the fowls were 9–10 weeks of age.

[3] Kligler, I. J., and Bernkopf, H., *Nature*, 1939, **143**, 899.
[4] Cox, H. R., *Pub. Health Rep.*, 1938, **53**, 2241.

2. *In Minced Embryonic Tissues in vitro.* Three series of cultures of this type were set up. In one, minced chick-embryo brain plus Tyrode's solution and 10% chicken serum served as medium. The second series was the same as the first, except that minced whole chick embryo was used in place of brain. In the last, River's medium was used: minced chick embryo in Tyrode's solution. In each instance the viral suspension, as well as 2 flasks of the inoculated culture, was titrated for determination of the viral titer at the outset. (Similar titrations of 2 or 3 culture flasks were carried out at the time of transfer.) The cultures were incubated at 36.5 to 37°C and transfers into the respective media were made at 5–6 days' intervals. Each series was carried through 5 passages, so as to exclude any possible dilution factor of the original viral inoculum. The results are summarized in Table I and reveal that in the culture of whole-embryo tissue

TABLE I

Viral Activity in Various Minced Chick Embryo Media

Titer of stock virus used for seeding cultures	Media	Titer of virus + media immediately after set-up	Subplants				
			1st	2d	3d	4th	5th
10^{-5}	Brain + serum	10^{-3}	10^{-3}	$<10^{-2}$	0	0	0
10^{-5}	Embryo + serum	10^{-3}	$>10^{-4}$	10^{-2}	10^{-4}	10^{-4}	10^{-2}
—	Embryo + Tyrode's solution	10^{-4}	$>10^{-2}$	10^{-2}	0	0	0

plus serum, there was a viral titer of 10^{-3} at the outset and at least 10^{-2} in the 5th subplant, although the original virus itself was calculated as being diluted to 10^{-8} in this passage. In the other 2 series the virus was detectable only through the 2d subplant; this corresponds to the point beyond which the virus is diluted out of its original activity.

Again it is clear that the avian virus has distinctive requirements for multiplication, such as are not found to exist for several others. Multiplication took place in minced whole embryos suspended in serum-Tyrode-solution mixture. The brain cultures were negative after the 2d subplant, as were those made with minced embryonic tissue in Tyrode's solution without serum.

It would appear, therefore, that the virus is rapidly lost at 37°C unless embryonic tissue and serum are present.

Summary and Discussion. 1. Embryonic chicks are apparently not susceptible to infection with this avian virus, although birds just hatched are. This is a strikingly reversed state from that which prevails in the case

of certain other viruses,[5] which multiply readily in the undifferentiated tissues of the developing embryo but are inactive in the hatched chick. Whatever the reason may be, a fact of epidemiological significance emerges: the disease-agent is probably not transmissible by way of the egg, thus supporting the prior finding of Jones.[1]

2. The virus multiplied in minced whole-embryo tissue-cultures *in vitro* only under certain indicated conditions. The method[8] in its present state, however, is not favorable for obtaining large yields of highly potent virus for use in immunizing procedures. No multiplication of virus was noted in this medium when chick-embryo brain was used instead of whole-embryo tissue. It is of interest in this connection that mammalian embryo-brain cultures have been found suitable for the multiplication of the neurotropic viruses of poliomyelitis[6] and of rabies.[7]

[5] For a discussion and references, see: Rivers, T. M., and Schwentker, F. F., *J. Exp. Med.*, 1932, **55**, 911; and Mackenzie, R. D., *J. Path. and Bact.*, 1933, **37**, 75.

[6] Sabin, A. B., and Olitsky, P. K., PROC. SOC. EXP. BIOL. AND MED., 1936, **34**, 357.

[7] Kanazawa, K., *Japanese J. Exp. Med.*, 1936, **14**, 519; Webster, L. T., and Clow, A. D., *J. Exp. Med.*, 1937, **66**, 125; Bernkopf, H., and Kligler, I. J., *Brit. J. Exp. Path.*, 1937, **18**, 481.

[Reprinted from THE JOURNAL OF IMMUNOLOGY, July, 1940,
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A STUDY OF IMMUNITY IN RABBITS FROM TWO TO THREE YEARS AFTER INFECTION WITH VACCINE VIRUS WITH ATTEMPTS TO RECOVER ACTIVE VIRUS

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(Received for publication, March 5, 1940)

There has been considerable discussion during the past decade of the hypothesis that immunity resulting from a viral disease depends on the persistence of virus in the host.

The problem has been stated and reviewed by a number of investigators, notably, Zinsser (1), Findlay (2), Traub (3), Lépine (4), and Rivers (5), who have cited many instances of the recovery of virus at remarkably long intervals after infection, and in some cases concomitant resistance to reinfection has been demonstrated. In order to establish the hypothesis, it is necessary not only to demonstrate the presence of virus in the immune host, but also to establish that the persistence of virus coincides with the duration of immunity. However, we need scarcely point out the danger of generalizing for all viral diseases, even though this relationship may be found to obtain in some.

Among the earlier observers,[1] Olitsky and Long (6) were able to recover vaccine virus by means of cataphoresis from the testes of an immune rabbit 114 days after intracutaneous inoculation of testicular virus. From another immune rabbit, virus was recoverable from the spleen at 133 days after inoculation but not from the skin or testes. They were unable to demonstrate virus from 133 to 178 days after similar inoculation, from the spleen or testes of 4 additional rabbits which proved to be non-immune. This relationship was further studied

[1] As early as 1887, L. Pfeiffer (quoted by Winkler (7) who also offers numerous examples of the persistence of protozoa and bacteria in relation to immunity) suggested, "mostly on clinical observation," that after recovery from smallpox, the immunity which develops is due to the survival of the virus within the body, *i.e.*, that man is immune to smallpox as long as he is a carrier of virus.

by Dresel (8), employing a large number of rabbits injected intracutaneously with testicular vaccine virus. By various methods he was able to recover virus regularly up to 252 days but not at 303 and 312 days. Tests for resistance to reinfection indicated that the strongest immunity occurred between 123 and 303 days, but was present up to 370 days. Dresel therefore demonstrated that, although immunity endured at least a year, virus was recoverable up to but not beyond 8 months after infection.

The present study was begun in this laboratory in 1935 by Dr. A. B. Sabin. He was able to demonstrate virus in the skin scar of a rabbit 3 months after infection, but not from the spleen of the same animal. In 3 other rabbits which he studied at 12, 14, and 14 months, respectively, he found a high content of neutralizing antibody in the serum, an active resistance to reinoculation, but no virus could be found in the testes of 1 rabbit or in the spleens of the other 2 animals. We continued the investigation[2] on these and other rabbits, and studied immunity 2 to 3 years after primary inoculation, correlating his findings with a renewed search for virus in various tissues, by a number of different methods to be described. This was as long an interval as possible since infection, for some were already showing signs of old age and were near the end of their life span under optimal animal house conditions. The serum of each rabbit was tested for the presence of agglutinins and precipitins, as well as for neutralizing antibodies. A systematic search for virus was made in the testes, spleen, and skin scar from each animal. From 2 animals, at death or by sacrifice, other organs were investigated as well. Some of the tissue suspensions were subjected to cataphoresis, but the majority underwent angle centrifugation which was found to be more effective for the purpose, as will be described later. The tissue suspensions were then transferred through two successive testicular passages in normal rabbits. Finally, each of the old vaccinal animals surviving was tested for residual active immunity. Details of materials and methods employed will be given in the appropriate section.

[2] We are grateful to Dr. Albert B. Sabin for offering these animals for study, and wish to acknowledge his results which he kindly made available to us.

MATERIALS AND METHODS

History of rabbits. Five male rabbits, referred to hereafter as rabbits or series 7-48, 7-49, 7-50, 7-51 and 7-53, had been injected July 1, 1936, intracutaneously in each of two sites with 0.2 ml of a 10 per cent suspension of testicular vaccine virus. Typical vaccinal lesions developed at each site, showing marked necrosis on the 4th day. These animals were bled on September 6, 1938. (Sterile precautions were used at all bleedings.) Spleen, skin scar, and a testis of each were removed at varying times between October, 1938, and May, 1939. Rabbit 7-49 died on March 16, 1939, of an intercurrent bacterial infection. The remaining 4 rabbits were tested for residual active immunity on May 1, 1939.

Rabbit 5-21 had been employed on February 2, 1935, for vaccine neutralization tests, as a result of which 16 vaccinal skin lesions developed. On February 7, 1936, both testes were removed for search for the presence of vaccine virus. On February 11, 1936, the animal was bled and tested for resistance to intracutaneous injection of virus. It was again bled on October 7, 1938, and was killed on December 30, 1938, after a period of increasing feebleness. The organs were removed and a suspension of each was preserved by freezing and drying in the Flosdorf-Mudd apparatus (9) until the test for the presence of virus was made. In accord with Rivers and Ward (10), we have found this procedure satisfactory for preserving vaccine virus.

Rabbit 38-04 had been used for vaccine neutralization tests on March 9, 1936, from which 8 cutaneous lesions developed, as well as generalized vaccinia. On June 10, 1936, the spleen and a skin scar were removed surgically. The animal was bled on October 7, 1938. Having become increasingly feeble, it died on December 24, 1938. The organs were kept in 50 per cent glycerol until tested for the presence of active virus.

Rabbits 5-13 and 5-15 each developed a single cutaneous vaccinal lesion. Each was bled, the spleen removed, and each animal subsequently tested for active immunity 14 months after vaccinal injection.

All rabbits which had undergone vaccinal infection were kept in individual cages in an isolation room throughout the remainder of their lives. By means of rigid isolation and careful handling of virus, we feel reasonably sure that chance infection with vaccine virus was eliminated.

Serum antibodies

Neutralizing antibodies. The serum of each rabbit bled after the interval indicated in table 1 was titrated for vaccinal neutralizing antibodies in the skin of normal rabbits, as follows:

The serum of each rabbit bled after an interval indicated in table 1 was titrated for vaccinal neutralizing antibodies. Tenfold dilutions of fresh testicular virus in 0.85 per cent sodium chloride were each mixed with an equal volume of test serum or normal control serum. After 1 hour's incubation at 37°C., each mixture was injected into the skin of 1, or in most instances, 2 normal rabbits.

From the results tabulated in table 1, it may be seen that the sera of 3 rabbits tested less than 1½ years after vaccinal infection neutralized 10,000 minimal skin doses (MSD). From 2 to 3 years after infection, the sera of rabbits in series 7-48 to 7-53 showed protection against from 10 to 1,000 MSD, and the sera of rabbits 5-21 and 38-04 against at least 10,000 MSD. It should be recalled that the latter 2 animals had responded to vaccinal injection with repeated or severe reaction; rabbits of series 7-48 to 7-53, with mild reaction.

It is clear that, after this prolonged period of from 2 to 3 years from the time of original exposure to vaccine virus, the rabbits' sera still exhibited virus-neutralizing antibodies, present, however, to a varying degree.

Agglutinins. The antigen was a vaccinal elementary body suspension[3] diluted in saline solution, buffered to pH 7.0 with phosphate-citric acid buffer (2 ml per 100 ml of saline solution) to give a faintly opalescent solution. The test was carried out according to the method of Craigie (11). Equal volumes of elementary-body suspension and twofold dilutions of serum were placed in a water bath at 50°C for 18 hours. A known immune and a known normal rabbit serum were included as controls, as well as saline controls of each test serum and of elementary-body suspension.

[3] We wish to thank Dr. Joseph E. Smadel for his kind coöperation, and for his samples of dermal filtrate and of suspensions of elementary bodies used in precipitative and agglutinative tests.

Of sera taken more than 2 years after vaccinal infection, those in series 7-48 to 7-53 showed no agglutinins; serum of rabbits 5-21 and 38-04, a titer of 1:16, and the control, known immune rabbit serum, 1:256. In sera taken within 6 months of vaccinal infection, agglutinin-titers of 1:320, 1:32, and 1:32 were found, respectively, in rabbits 5-21, 5-13, and 5-15 (table 1).

Although the number of observations is too small to form a basis for generalization, a low titer of agglutinins, as well as a high degree of virus-neutralizing antibodies, was observed in the sera of 2 rabbits which had undergone severe or repeated reaction to virus more than 2½ years previously, whereas agglutinins were not detected in the sera of those which gave a milder reaction a little over 2 years previously.

Precipitins. Following Craigie's method (11), dermal filtrate was diluted to a degree shown by preliminary test to give a sensitive reaction with positive serum. The diluent used was saline solution brought to pH 7.0 with phosphate-citric acid buffer (100 ml + 2 ml of buffer). Mixtures of equal parts of dermal filtrate and twofold dilutions of serum in buffered saline solution (making final dilutions of from 1:2 to 1:128) were incubated in a water bath at 50°C. for 18 hours.

No precipitins were found in the sera of the old vaccinated rabbits in a test in which known immune serum showed a precipitin-titer of 1:8 and hyperimmune, 1:64 (table 1).

Active immunity

Four rabbits of series 7-48 to 7-53 were available for final testing for residual, active immunity, carried out by titrating active virus in the skin and using 4 normal rabbits simultaneously as controls on the activity of the virus.

Into the clear, unpigmented areas of the clipped skin of the back and flanks of each rabbit, 0.5 ml of a suspension of freshly passaged testicular virus in tenfold dilutions of 10^{-3} through 10^{-8} was injected. Vaccinal lesions first appeared on the 2nd day, increasing in intensity up to the 5th day, after which secondary reactions interfered with readings. It was obvious that by the 5th day the type of response of most of the test rabbits was different from that of the normal control rabbits, as can be seen in table 2.

TABLE 1

Summary of experimental results

RABBIT NUMBER	REACTION TO INOCULATION OF VIRUS	SERUM ANTIBODIES				ACTIVE IMMUNITY		SEARCH FOR VACCINE VIRUS		
		Interval since last inoculation	Agglutinins	Precipitins	Neutralizing antibodies	Interval since last inoculation	Result	Interval since last inoculation	Tissues tested	Result
			titer							
7-48	2 cutaneous lesions	2 yrs., 2 mos.	0	0	MSD neutralized 1,000*	2 yrs., 10 mos.	0	2 yrs., 3 to 10 mos.	Spleen, testes, skin scar	0
7-49	2 cutaneous lesions		0	0	1,000		n.t.		Spleen, testes, skin scar	0
7-50	2 cutaneous lesions		0	0	10		>1†		Spleen, testes, skin scar	0
7-51	2 cutaneous lesions		0	0	10		10		Spleen, testes, skin scar	0
7-53	2 cutaneous lesions		0	0	10–100		10		Spleen, testes, skin scar	0
5-21	16 cutaneous lesions	6 mos.	1:320							
		1 yr.		n.t.	10,000	1 yr.	>5,000	1 yr.	Testes	0
	Skin test 1 yr. later	2 yrs., 8 mos.	1:16	0	>10,000	n.t.		2 yrs., 10 mos.	Other tissues‡	0
38-04	8 cutaneous lesions with marked generalized reaction	2 yrs., 7 mos.	1:16	0	>10,000	n.t.		3 mos.	Skin scar, spleen	+ 0
								2 yrs., 10 mos.	Other tissues‡	0
5-13	1 cutaneous lesion	6 mos.	1:32	n.t.		1 yr., 2 mos.	>100,000	1 yr., 2 mos.	Spleen	0
		1 yr., 2 mos.			10,000					
5-15	1 cutaneous lesion	6 mos.	1:32	n.t.		1 yr., 2 mos.	>100,000	1 yr., 2 mos.	Spleen	0
		1 yr., 2 mos.			10,000					

* MSD = minimal skin doses; *i.e.*, titer of virus + normal serum = 10^{-6}; end point of virus + test serum = 10^{-2}; MSD = 1,000 MSD neutralized.

† See table 2 for degree of immunity.

‡ See text. N.t. = not tested.

TABLE 2

Resistance of rabbits to intracutaneous injection of vaccine virus 2 years and 10 months after infection

DILUTION OF VIRUS	5TH DAY		7TH DAY	5TH DAY		7TH DAY	5TH DAY		7TH DAY	5TH DAY		7TH DAY
	Size	Lesion	Lesion	Size	Lesion	Lesion	Size	Lesion	Lesion	Size	Lesion	Lesion
	Test rabbits											
	No. 7-48			No. 7-50			No. 7-51			No. 7-53		
	cm			*cm*			*cm*			*cm*		
10^{-3}	6 x 4	HN	HNe	2.5 x 2	HE	hRe	2 x 2	RE	HRE	3 x 1.5	RE	R
10^{-4}	3 x 2.5	HN	HNe	1.5 x 1.5	R	RT	1.5 x 1	RE	R	3 x 1.5	RE	R
10^{-5}	2 x 1.5	HN	HN	0.5 x 0.5	R	Rt		—	—		—	—
10^{-6}	1 x 1	H	n		—	—		—	—		—	—
10^{-7}	0.1 x 0.1	t	—		—	—		—	—		—	—
10^{-8}		—	—		—	—		—	—		—	—
	Control rabbits											
	10			20			21			22		
10^{-3}	4.5 x 3.5	HNE	HNe	6 x 4	HNE	HNe	7 x 4	HNE	HNE	5 x 4	HNE	HNe
10^{-4}	4 x 3	HNE	HNe	4 x 2	HNE	HNe	3 x 2	HNE	HNE	1.5 x 1.5	RN	Ne
10^{-5}	2 x 2	hnE	HNe	1 x 1	HE	hne	1.5 x 1.5	RE	HNe	1 x 1	Rn	hNe
10^{-6}	1.5 x 1.5	hnE	HNe	0.5 x 0.5	R	he	1 x 1	RE	he		—	—
10^{-7}	1 x 1	Ht	e		—	—		—	—		—	—
10^{-8}		—	—		—	—		—	—		—	—

H = hemorrhage; N = necrosis; E = edema; R = erythema; T = nodule; small letters = slight reaction of character indicated. — = negative.

In table 2 may be seen the results of titrations of vaccine virus carried out simultaneously in the 4 test rabbits, 2 years and 10 months after vaccinal infection, and in 4 control rabbits. The latter were obtained from the animal-house stock and were between 1 and 2 years of age. There was more variation in the response of the controls, which showed titers of 10^{-7}, 10^{-6}, 10^{-6}, and 10^{-5} respectively, than usually encountered. Since the original source of each rabbit was not known to us, we cannot state positively that there had been no previous contact with vaccine virus, as perhaps suggested by the results in rabbit 22. Of the test rabbits, 7-48 exhibited a slight nodule at 10^{-7} dilution, with hemorrhage and necrosis in the reactions through 10^{-6} dilution. This animal was regarded as non-immune. It is of interest to note that serum of rabbit 7-48 neutralized 1,000 MSD 2 years and 2 months after vaccinal infection and that 8 months later the animal was not resistant to cutaneous reinfection. Rabbit 7-50 showed hemorrhage and edema at 10^{-3} dilution, and erythema and nodule formation through 10^{-5}. This was interpreted as a slight immunity. In rabbits 7-51 and 7-53, the reactions of erythema and edema appeared at 10^{-3} and 10^{-4}. Thus, these rabbits showed a moderate degree of resistance to reinfection.

Since the test animals were over 2 to 3 years of age and had been castrated from 18 to 22 days prior to the immunity test, while the controls were 1 to 2 years old and had not been castrated, the following experiment was performed to ascertain the bearing of the factors of age and castration on susceptibility to infection with the virus.

One of 2 rabbits 1 to 2 years of age, and one of 2, 2 to 2½ years of age,[4] were castrated. Three weeks later vaccine virus was titrated intracutaneously in all 4 animals. No difference in titer was observed (10^{-6} in all instances). The evidence indicated that in these experiments the ages of the rabbits used and castration were not essential factors in the difference in reactions exhibited by the test rabbits and by the controls.

[4] These were the oldest rabbits available and we wish to thank Dr. Louise Pearce for placing them at our disposal.

Thus, it was shown that, of the 4 rabbits subjected to a test for active immunity 2 years and 10 months after injection with vaccine virus, 1 was not immune, 1 had a slight, and the other 2, a moderate degree of resistance to reinfection.

Search for active virus in tissues

a. *Selection and treatment of tissues.* The choice of tissues to be tested for the presence of active virus was limited since, after their removal, it was desirable that the animals be kept alive for a final test for active immunity.

The spleens of rabbits 5-13 and 5-15 were removed 1 year and 2 months after vaccinal infection. Ether anesthesia was used and sterile precautions were observed throughout. After cataphoresis, samples were injected into normal rabbits. From each of the 5 rabbits of series 7-48 to 7-53 spleen, testis and skin scar (from the original vaccinal lesion) were removed. The tissues were excised after an interval of from 2 years and 3 months to 2 years and 10 months after the original vaccinal inoculation. Each tissue, immediately after removal, was ground with alundum; a saline suspension of each (with two exceptions) was subjected to angle centrifugation and then passaged serially in rabbits, as described later. In one instance, a suspension of skin scar was injected directly; in another, similar material was subjected to cataphoresis. The testes of rabbit 5-21 had been removed 1 year after the last vaccinal inoculation. A suspension was transferred directly, as well as after cataphoresis, to a normal rabbit, by intracutaneous, intracerebral, and intratesticular routes. Rabbit 5-21 was killed 2 years and 10 months after the last vaccinal inoculation. The following tissues were removed and kept in the frozen and dried state: brain, lung, skin scar, spleen, and adrenal. From 2 to 6 weeks later, they were studied for the presence of virus by animal injection. A suspension of each was then subjected to angle centrifugation and passaged twice, as described below. Three months after the original vaccinal infection, spleen and skin scar had been removed from rabbit 38-04; a suspension of each, after cataphoresis, was inoculated intratesticularly into 2 successive, normal rabbits. Rabbit 38-04 died 2 years and 10 months after the original vaccinal inoculation. The following organs were removed after death: brain, adrenal, and kidney. They were placed in 50 per cent phosphate-buffered glycerol and kept for several months, then treated

by cataphoresis and passaged intratesticularly through 2 normal rabbits in series.

b. *Method of cataphoresis:*

The cataphoresis apparatus of Todd (12), as adapted by Olitsky and Long (6), was employed. This consists of a U-tube connected by saline-agar bridges with saline solutions and, in turn, with copper sulfate solutions, from which copper electrodes carry the current of 110 v.-10 amp. D.C. house circuit. The tissue was ground with alundum and suspended in 30 ml of phosphate buffer at pH 6.7 and then placed in the U-tube. A current which fell from 6 to 3 milliamperes flowed through the suspension for 3 hours. Samples after cataphoresis were removed from the anodal and cathodal ends of the U-tube, as well as from anodal and cathodal tips of the agar bridges, the agar samples being ground in a minimal amount of saline solution. Of each sample, 0.5 ml were injected intracutaneously in a normal rabbit, and 1 ml each of anodal and cathodal agar suspension, intratesticularly as well. In most instances a suspension of the testis which had received anodal agar was injected 6 or 7 days later into the skin and testis of a second-passage rabbit.

Results of cataphoresis. Vaccine virus was not recovered from any of the tissues examined more than 2 years after the last exposure to virus (table 1), even though the tissues, after cataphoresis, were transferred through 2 successive normal rabbits. Virus had been demonstrated, however, from a skin scar, although not from the spleen of rabbit 38-04 after an interval of 3 months from the time of the initial cutaneous inoculation of vaccine virus. In this instance, an excised dermal scar, treated by cataphoresis, evoked no response after testicular injection into a normal rabbit, but the testis of the latter brought about in a second normal rabbit characteristic vaccinal lesions in the skin and testes. On the other hand, no vaccine virus had been recovered from the spleen of rabbits 5-13 and 5-15, 1 year and 2 months after vaccination, and none from both testes of rabbit 5-21 one year after last vaccinal injection, when inoculated before and after cataphoresis into a normal rabbit by the intracutaneous, intratesticular, and intracerebral routes.

It was then thought desirable to reinvestigate cataphoresis, as used, as a method for recovering small amounts of active virus from tissue suspensions known to contain neutralizing antibodies.

Applicability of cataphoresis. In order to ascertain whether our method of cataphoresis was an effective means for freeing small amounts of virus in an active form from a mixture of virus and immune serum, the following series of experiments was undertaken.

First, in order to determine a "just neutral" mixture, titrations by intracutaneous injection in rabbits were made of mixtures of a constant amount of immune serum with tenfold dilutions of virus in saline solution. This point was chosen as the most favorable for the recovery of virus. After cataphoresis of such a mixture, the anodal agar, cathodal agar, anodal fluid and cathodal fluid, and, as a control, the untreated mixture were tested for viral activity by intracutaneous injection in a normal rabbit. Active virus was not recovered at the anode when the mixture proved neutral. When, on the other hand, this mixture, neutral in the preliminary titration, was used as control on the cataphoresis material and proved to be active in a fresh rabbit, then the anodal agar suspension was also active. This individual variation indicates the importance of controlling the neutrality of a given mixture: the "neutral" mixture must be tested in the same rabbit which is injected with cataphoresis materials in order that the demonstration of active virus may be significant.

It was shown, in accord with Dresel (8), that this method of cataphoresis does not fulfill the requirements for the present study. We turned, therefore, to the method of angle centrifugation.

Angle centrifugation. It has been reported by Sabin (13) and others that active vaccine virus can be separated from a neutral immune serum-virus mixture by horizontal centrifugation at high speed. That vaccine virus was readily recoverable from a neutral mixture by centrifugation in an angle centrifuge was shown by the following.

A mixture containing 100 minimal skin doses of virus (titer, 10^{-7}) in hyperimmune serum at a final dilution of 1:10, was kept for 1 hour in the refrigerator. One part of the mixture was then placed at room

temperature as a control, while another portion was centrifuged at 5,000 rpm. The sediment obtained was washed with M/100 phosphate buffer at pH 7.8 and again spun at the same rate for 1 hour. The resuspended sediment produced marked, typical vaccinal lesions when injected intradermally into 2 normal rabbits; the control neutral mixture gave rise to no vaccinal lesions when injected into the same rabbits, nor did the first supernate after angle centrifugation.

It was found, therefore, that by means of angle centrifugation active vaccine virus could be readily recovered from a neutral, immune serum-virus mixture. The remaining tissues were then studied in the following manner.

The tissue to be tested for virus was ground with alundum and suspended in 5 ml of phosphate buffer. After horizontal centrifugation for 20 minutes at 2,000 rpm, the supernate was spun at 5,000 rpm in an angle centrifuge. The supernate was removed and the sediment resuspended in 2 ml of phosphate buffer. The supernate and the sediment were each injected into a normal rabbit, 0.5 ml each into the skin and 1 ml each into a testis. The testis injected with sediment was passaged after 6 or 7 days into the testis and skin of another normal rabbit.

No active virus was recovered by means of angle centrifugation and intratesticular passage through 2 successive rabbits (table 1). The tissues tested were skin scars, spleens, and testes derived from rabbits of series 7-48 to 7-53, as well as skin scar, spleen, brain, lung, and adrenal removed after sacrifice of rabbit 5-21, and brain, kidney, and adrenal of rabbit 38-04 after death. These were obtained from the rabbits after an interval of nearly 3 years from the last intracutaneous injection of vaccine virus.

Results of the study of immunity and search for virus are summarized in table 1.

DISCUSSION

In a study of 2 to 3 years' duration, such as is presented in the present communication, there is the inherent difficulty of keeping animals in good condition, and in isolation to prevent accidental infection. Withal, the number of animals that could reach the final tests was small. Furthermore, since it was de-

sirable to save the rabbits for a subsequent test for immunity, the choice of organs or tissues to be excised for the study of the presence of virus was limited.

The results show that after a period of almost 3 years from the time of vaccinal infection, some rabbits exhibit resistance to reinfection (in 3 of 4 tested). Neutralizing antibodies were demonstrable to a varying degree in all 7 sera tested, and agglutinins in 2 of the 7 sera. From 2 to 3 years after infection, and in 3 rabbits after 1 year, it has not been possible, however, to demonstrate active virus. From one of the rabbits, virus had been demonstrated after 3 months. In an earlier study, Olitsky and Long (6) reported recovery of active virus from 2 rabbits after 114 and 133 days, respectively, but not from another animal at 133 days, nor from 3 others from 143 to 178 days. Dresel (8), in a large series of rabbits, recovered virus regularly up to 252 days but not at 303 and 312 days. Hence, the inability to recover virus after about 2½ years is not inconsistent with previous reports. The former investigators (6) found the animals from which no virus had been recovered to be non-immune, as tested by intracutaneous inoculation of virus. However, there were no data comparable with the serum-neutralizing antibodies as reported in the present communication. It should be added at this point that Dresel demonstrated resistance to reinfection up to 370 days. Hence, the longest interval thus far reported on duration of vaccinal immunity in rabbits has been extended by the present findings.

A significant point is the recovery of virus from a skin scar 3 months after initial infection—at a time when one could, in view of a later demonstrated immune response, consider the animal immune. How this is related to the negative findings at later periods is conjectural. It should be emphasized, however, that we can speak only of presence or absence of detectable virus. It is not impossible that the quantity of virus has been so reduced that recovery of a single infective unit in a given sample of tissue may be difficult, or that the residual virus has been masked by antibodies to a degree beyond recovery by the methods employed.

In conclusion, the present report indicates that immunity to

vaccine virus may persist in certain instances for from 2 to 3 years, *i.e.*, into old age of a rabbit, when, by the methods used, virus is no longer demonstrable in the tissues. By the means employed in this investigation, vaccinal immunity has been shown to endure beyond the persistence of recoverable virus.

SUMMARY

1. Of 4 rabbits tested for active immunity nearly 3 years after vaccinal infection, 1 was not immune, 1 showed a slight, and 2 a moderate degree of resistance to cutaneous reinfection.

2. No active virus was recovered from 2 to 3 years after vaccinal infection from selected tissues of the 7 rabbits studied. From the testes of 1 of these animals and the spleen of 2 others, no virus had been detected after 1 year. However, from a skin scar but not from the spleen of 1 rabbit, vaccine virus had been recovered 3 months after infection.

3. Sera from the 7 rabbits, about 2½ years after the last vaccinal infection, neutralized from 10 to 10,000 minimal skin doses of vaccine virus.

4. Sera from 2 of the 7 rabbits, more than 2½ years after last infection, showed agglutinins to vaccinal elementary bodies to a titer of 1:16; the other 5 sera showed none. No precipitins to dermal filtrate were found in any of the sera.

5. The results are discussed with reference to the problem of persistence of virus and duration of immunity.

ADDENDUM

After this paper had been submitted for publication, an article by J. M. Pearce (Jour. Inf. Dis., 1940, **66**, 130) appeared on the relationship of vaccinal immunity to "latent" virus. The results reported agree essentially with those reported here.

REFERENCES

(1) Zinsser, H.: Immunity Principles and Application in Medicine and Public Health. By H. Zinsser, J. F. Enders, and LeR. D. Fothergill, The Macmillan Co., New York, 5th edition, 1939, p. 443.

(2) Findlay, G. M.: Brit. Med. Jour., 1939 (Feb. 11), **1**, 257.

(3) Traub, E.: Ztschr. f. Infektionskrankh. Haustiere, 1939, **54**, 169; Jour. Exper. Med., 1936, **63**, 847; 1939, **69**, 801.

(4) Lépine, P.: Rev. d'immunol., 1939, **5**, 350.
(5) Rivers, T. M.: Lane Medical Lectures: Viruses and Virus Diseases, Stanford University Press, 1939, Med. Ser. IV, No. 1.
(6) Olitsky, P. K., and Long, P. H.: Jour. Exper. Med., 1929, **50**, 263.
(7) Winkler, W. F.: Ergebn. allg. Path. u. path. Anat., 1925, **21**, pt. 1, 45.
(8) Dresel, E. G.: Ztschr. f. Immunitätsforsch., 1932, **75**, 337.
(9) Flosdorf, E. W., and Mudd, S.: Jour. Immunol., 1935, **29**, 389.
(10) Rivers, T. M., and Ward, S. M.: Jour. Exper. Med., 1935, **62**, 549.
(11) Craigie, J.: Brit. Jour. Exper. Path., 1932, **13**, 259.
(12) Todd, C.: Brit. Jour. Exper. Path., 1927, **8**, 369.
(13) Sabin, A. B.: Brit. Jour. Exper. Path., 1935, **16**, 70.

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A TRANSMISSIBLE AGENT (THEILER'S VIRUS) IN THE INTESTINES OF NORMAL MICE

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Spontaneous encephalomyelitis of mice—Theiler's disease—is of considerable interest from at least two points of view: its widespread, geographic distribution and its resemblance in several characteristics to poliomyelitis of man. The disease was first observed by Theiler (1), and later by Sabin and Olitsky (2) among Rockefeller Institute (2) and Swiss (1) strains of stock albino mice; then its existence in Germany (3) was reported, later in Japan (4), and recently in Palestine (5). Its similarity to poliomyelitis is striking; it is now commonly designated as "poliomyelitis of mice" (4). The features of the two maladies having closest resemblance are size and thermal inactivation of their causal agents, action on the central nervous system, the pathological changes induced, the clinical reactions, and certain epidemiological factors.

The size (9 to 13 mμ) of the causal agent of the spontaneous disease of mice (1, 9) is practically the same as that of the poliomyelitis virus. This is especially significant since the size is in the minimal range for viruses and thus far only one other virus as small has been encountered, namely, that of foot-and-mouth disease. In addition, the writer has shown that thermal inactivation (50–55°C. for 30 minutes) of the active agent of mouse encephalomyelitis, as present in the brain or intestinal contents, takes place at about the same temperature required to inactivate poliomyelitis virus (6).

The action of both viruses is chiefly on the central nervous system. The mouse agent induces lesions only in the nervous system (1) and it is from the spinal cord that the maximum amount of virus can, as a rule, be recovered (1, 4). The lesions of neuronal necrosis, neuronophagia, perivascular mononuclear cell infiltration, and glial cell proliferation, occurring mostly in the cord and in the anterior horns thereof (1, 4); the sparseness of reaction in the cortex, taken together with the slight or absent meningeal involvement (4), are similar in nature to the pathological changes of poliomyelitis in man and the experimental disease in the monkey.

The clinical picture of Theiler's disease is in many respects like that of poliomyelitis, especially in the occurrence of the characteristic flaccid paralysis of the extremities, associated with a fairly rapidly developing muscular atrophy and followed by contractures and deformities (1, 4). Furthermore, the nervous signs arise after a period of incubation of from 5 to 7 to over 30 days and have an average duration of 10 days (4).

A certain degree of likeness exists in the incidence of the two maladies. The mouse

affection has been estimated to occur in one of 2,000 (1) or one or two of 1,000 (2) purchased or stock animals. In the writer's recent series of over 5,000 stock mice examined —normal ones and those inoculated with different materials by various routes—not a single, clinically apparent case of Theiler's disease was observed to arise spontaneously. Another similarity is noted between poliomyelitis and the experimental Theiler's disease: susceptibility to both infections depends on age. Almost all suckling mice die without visible signs of paralysis, but with increasing age the incidence of paralysis increases and that of mortality decreases, to about 6 or 7 weeks of age; in older mice there is generally a greater resistance to inoculation (1). The resistance has been ascribed (1) to an immunity which is built up gradually by older animals, although Theiler, and Theiler and Gard (7) have not shown definitely as yet that production of increasing viral neutralizing bodies is the basis. The influence of increasing age on the increased incidence of antibodies and of resistance to clinically apparent poliomyelitis in man is now well known, although the reason for the resistance of older persons may rest on the different grounds of physiological or structural barriers which may develop with age (*cf.* Sabin and Olitsky, 8).

Finally, attention is called to the fact that the two diseases were found, in earlier experiments by Theiler (1), to be distinct, with no relationship between them in host susceptibilities and immunological reactions. A final word on this subject is, however, still to be said, and the problem is now being studied anew by Theiler and by the writer.

However the problem of the relationship of the two diseases is solved, the malady of the mouse is one that may be profitably investigated, especially since the virus is not recoverable from the blood but is obtained regularly from the central nervous system (1, 4) and, as recently determined (7, 11, 12), also from the intestinal tract, and it might be hoped that facts got therefrom can be carried over to studies on poliomyelitis. The advantages of such a procedure are plain: there exists the difficulty in poliomyelitis research of having ample and regular supplies of monkeys at hand and their cost is high; moreover, experimental poliomyelitis is not a natural disease of the monkey, whereas Theiler's disease is indigenous in the mouse.

Thus we have shown the grounds for our interest in spontaneous encephalomyelitis of mice[1] and in view of the current discussion on the presence of virus in the feces of patients having poliomyelitis, and of healthy contacts with the disease, as well as in sewage originating from epidemic areas (6, 10), it was thought desirable to study the intestinal contents of mice having the Theiler-disease syndrome.

In two recent brief papers (11, 12) it was reported that at the outset of this study (11), brain, also the intestinal contents, of a mouse the only visible sign in which was circling gait, were injected intracerebrally—the brain in four normal mice and contents in four others. Only one of the eight developed encephalomyelitis resembling Theiler's disease,

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[1] The writer is grateful to Dr. Max Theiler of the International Health Division of The Rockefeller Foundation for his cooperation.

and that one had received intestinal contents. In view of the fact that the source of the material was a mouse, the only sign in which was circling, and the brain of which at autopsy contained no virus, the animal could not be considered as having had Theiler's disease at that time. However, the single positive result was considered significant and the first experiment was followed by several others in which were investigated the brain and intestinal contents of mice having characteristic Theiler's disease, either the spontaneous or the experimental form. The results of the next four series revealed that in each test the paralyzed mice harbored a readily transmissible active agent resembling the virus of Theiler's disease, not only in the brain but also in their intestinal contents.[2] From this point studies were continued with normal mice. The latter, as well, were found to harbor this transmissible active agent in their intestinal contents.

It was shown (12) that the encephalomyelitis-producing agent was not detectable in the intestines of normal mice from the fetal stage to 12 days of age, and this was true regardless of its presence in or absence from the mothers' intestines. After weaning, however, at 20 to 25 days of age, it was demonstrable, although irregularly. Every experiment, then, of the eighteen undertaken with mice 30 days old, in which were tested the contents of one mouse or pooled material from two to five normal animals, usually three, yielded positive results. Thus, of 130 mice injected intracerebrally with the filtered intestinal contents derived from 60 normal mice, 123 developed signs resembling Theiler's disease. On the other hand, in old normal mice (6 months or older in age), the virus was still present but was recoverable with less regularity than from the young adults. It is of interest that it was not detected in the brains of normal mice (thirty-two were so examined) even though the same animals harbored it in their intestinal contents.

Preliminary experiments were undertaken (12) with the object of determining a cause for the absence of the active agent from the intestines of normal, unweaned mice up to the age of 12 days. It was found that the mothers' milk had no neutralizing capacity, nor did it contain the active agent; that pregnant animals purposely brought down with encephalomyelitis by means of intracerebral inoculation, bore young that, at the age of 1 to 5 days, were free of the infective material.

With respect to the problem of the presence of the intestinal agent after weaning, it was demonstrated (11) that the animals' food—both fluid and solid—was uncontaminated by the virus. Later, Theiler and Gard (7) revealed, however, that feces passed by normal mice contain the encephalomyelitic agent as do the intestinal contents,—a fact which we readily confirmed. The problem then turned towards determining whether food contaminated with the infective agent present in feces could bring about a persistent carrier state in the weaned and older mice. Theiler and Gard (7) have investigated the presence of the virus in feces: stock mice 6 weeks of age, kept in special platform cages to avoid fecal contamination and given sterile food, passed feces which were continuously infective for at least 53 days. The further problem, whether feces-contaminated food can initiate the infection, is still to be solved (9).

Finally, mention has already been made (7, 11, 12) of the fact that cross-active immunity exists between the mouse-passaged disease, induced by intracerebral injection of nervous tissue originally derived from spontaneous encephalomyelitis, and the infec-

[2] The writer is indebted to Dr. A. B. Sabin and Dr. Max Theiler for the use of several mice ill with spontaneous or experimental encephalomyelitis.

tion brought about by intracerebral inoculation of filtered intestinal contents secured from normal mice. It is therefore justifiable to conclude that the active agent in the latter material is a strain of Theiler's virus or is closely related thereto, especially since its properties in general are indistinguishable from those of the latter (7, 11, 12). The active agent found in normal mouse intestines will, for present purposes, be called "intestinal virus."

In this paper, the results of the experiments thus far reported (11, 12) on the intestinal virus will be elaborated and certain of its other properties will be described, including its relation to the virus that induces spontaneous Theiler's disease.

EXPERIMENTAL

Methods and Materials.—To obtain virus for experimental purposes, the intestinal contents of 30- to 40-day-old albino mice of the Rockefeller Institute strain were used. The contents of from one to five mice—pooled when more than one was available—were expressed from a varying length (usually 10 inches) of the small intestine and ground with sand in a mortar, in plain broth or in saline solution when the material was intended for animals other than mice. The suspension made up to from 5 to 10, usually 8 per cent, was spun in a horizontal centrifuge at 2,500 R.P.M. for 10 to 20 minutes and the supernatant fluid passed once through a small Berkefeld V filter, or through a single-pad, small Seitz filter. The filtrate, after being seeded in broth or on agar media, rarely yielded growth of bacteria supposedly of intestinal origin; nevertheless, the intracerebral inoculation of such "contaminated" filtrates did not, as a rule, interfere with the specific effect; the occasional reaction due to the cultivable bacteria could be easily identified as such. Five to fifteen, usually six to ten albino mice 20 to 40 days of age were used for each series of tests and the dosage for intracerebral inoculation was 0 03 cc [3] For subsequent transfer, either the brain and cord, or again the intestinal contents of an animal showing signs of experimental encephalomyelitis, were employed successfully. Inoculated animals which failed to react within 40 days were designated as negative.

The diagnosis of the specific experimental disease was based upon the presence of (*a*) flaccid paralysis which developed from 7 to 37 (usually 10 to 20) days after inoculation; (*b*) characteristic histopathological lesions in the central nervous system[4] and lack of changes in other tissues; (*c*) positive transmission to fresh mice, by cerebral route, of 10^{-1} dilution of brain and spinal cord (or cord alone in animals which had been paralyzed for a period longer than 5 days); and finally (*d*) the demonstration of cross-immunity in animals convalescent from spontaneous Theiler's disease and from experimental intestinal virus infection.

Age of Carriers of Intestinal Virus.—In the preliminary communication (12) it was shown that fetal and suckling mice tested up to the 12th day were

[3] All operations on animals were made with the aid of full ether anesthesia.

[4] We have also found that an occasional neuron shows Cowdry type B intranuclear inclusions, similar to those seen in human and monkey poliomyelitis (6), in the early stages of the encephalomyelitis induced by filtrates of intestinal contents

free of intestinal virus; those 20 to 25 days of age revealed its presence irregularly, 30-day-old ones invariably, and old mice (6 months or older), again irregularly. There was a wide gap of unrecorded instances of the incidence of intestinal virus in the ages between 30 days and 6 months or over. The records of some of these ages and also those of additional tests with 30-day-old mice are presented in Table I.

It will be observed from the table that 30-day-old animals, including those at varying ages up to about 7 weeks, harbored virus in the intestinal contents, a 10^{-1} dilution of which, on transfer intracerebrally to other mice, exhibited a high percentage of incidence of encephalomyelitis. On the other hand, intestinal filtrates of mice, 51 to about 100 days of age, while yielding virus in every trial, showed a lower percentage of incidence of paralysis in inoculated mice. The decreased incidence in the latter series can probably be ascribed either to a gradual loss of viral content in the intestines with increasing age, or to inclusion in the pooled samples of each test of the contents of an older mouse which at that age may have been free of virus. In this connection, the finding of Theiler and Gard (7), that with time a certain number of mice cease to be carriers, is supported. It is also plain that feces contain the virus (Table I), a fact first demonstrated by the latter observers (7), and that apparently normal stock mice, as well as those paralyzed during the course of experimental encephalomyelitis, are carriers. Furthermore, no distinction can be made between the effect of material derived from well and that from paralyzed animals—contents from normal mice and from paralyzed ones were active to the same end-point of dilution, 10^{-3}.

If the numerical results now presented are added to those already recorded (12), a striking fact emerges. Thus in the combined series, comprising 47 experiments on mice 20 days of age or older, in which 167 normal stock animals were used as sources of intestinal contents (or feces), all but three of the tests were positive for the presence of virus; of the latter three, two were made with the contents of mice 20 days old, and one with material from animals 6 or more months of age. Still more striking is the fact that in this combined series 33 experiments were made with the intestinal contents or feces derived from 124 normal mice of an age from 30 to 49 days; not only were all the individual tests positive, but of 255 mice inoculated with the material, 237 came down with the paralysis of experimental encephalomyelitis—a case incidence of about 93 per cent.

The 10^{-1} dilution of filtered intestinal contents which was employed would represent an amount of virus which is probably 10 to 100, or even less, infective cerebral doses. In repeated tests on titration of the contents

TABLE I

Age at Which Intestinal Contents of Normal Mice and Those Having Encephalomyelitis Contain Virus (Data Additional to Those Already Reported (11))

Experiment	Source mice: No.	Source mice: Age	Source mice: History	Test mice, contents injected intracerebrally: No. (30 days old)	Test mice, contents injected intracerebrally: No. paralyzed	Remarks
		days				
A to G	26	30	Normal stock	60	53	Summary of 7 different tests with pools of 3 to 5 mice in each
A	10	35	" "	10	8	Only feces used—collected over a period of 3 hrs.
A	5	36	" "	7	7	
A	2	37	" "	10	9	
A	3	40	" "	10	9	
B	5		" "	6	6	
A	4	42	" "	6	6	
B	4		" "	10	10	
A	3	46	Paralyzed 2 to 4 days	7	7	Intestinal virus inoculated intracerebrally in source mice
A	2	48	Paralyzed 1 and 3 days	6	6	
A	5	49	Normal stock	6	6	
A	3	51	Paralyzed 1 and 2 days	8	5	Spontaneous Theiler's disease of source mice
A	2	60	Normal stock	8	5	
A	3	67	" "	6	4	
A	3	About 100	Paralyzed 43 days	8	4	171st passage of spontaneous Theiler's disease in source mice. Brain and cord of source mice negative for virus

for viral activity by the intracerebral route, the end-point was determined at not higher than 10^{-2} to 10^{-3} dilution; the end-point of activity of tissue of the central nervous system of mice during the active stages of encephalo-

myelitis caused by Theiler's or intestinal virus is, by intracerebral test, about 10^{-4}. Further experiments on quantitative relationships are, however, now being carried on.

What is here disclosed is the extraordinary distribution of the intestinal virus in normal mice—it is almost invariably found in all animals of young adult and mature life. Moreover, it is present then in the intestinal contents in an appreciable quantity, for it is clear that in such material there must be a considerable amount of inert solid and fluid, and little cellular substance.

Source of Intestinal Virus.—

Theiler and Gard (7) have presented evidence to show that the persistence of intestinal virus in normal mice is not due to continuing contamination from some outside agency but to some condition present in the mouse itself. We have demonstrated (12), as have also the writers just mentioned (7), that no virus is found in the central nervous system of normal mice. Nor is virus recoverable from their salivary glands or nasal mucosae (11), and in a repetition and extension of the latter experiments, none was detected in any part of the head of a normal mouse (7). Theiler and Gard therefore concluded from these and other results that the source of infection is probably the small intestine and not a point further up in the alimentary canal.

Gall-Bladder and Pancreas.—Since the gall-bladder and pancreas have direct connection with, and excrete material into the intestinal tract, attempts were made to determine whether the organs contained virus. In duplicate experiments, in which eight normal 30- to 40-day-old mice were employed, pooled intestinal contents of which yielded virus as was to be expected, neither gall-bladder (nor bile) nor pancreas, prepared and injected intracerebrally in thirty-six mice, gave rise to any viral effect.

Mesenteric Glands.—

The mesenteric glands were removed surgically from normal mice or those paralyzed after intracerebral inoculation of filtered intestinal contents derived from normal animals. Suspensions of the glandular tissue in broth in concentrations of 1:2 to 1:10 were prepared and these were injected intracerebrally in 30-day-old mice. The details of the various experiments are summarized in Table II.

It will be noted from the tabulated results that in five experiments only four mice were brought down with encephalomyelitis, although 59 were inoculated with the mesenteric glands culled from fifteen mice. Theiler and Gard (7) reported somewhat similar results—though even a lower case incidence of paralysis was induced in their trials. Moreover, it is apparent that in the present series of tests the practical limit of dilution

for inoculability (1:2) and for infectivity was reached. One is justified in concluding, therefore, that small amounts of virus can be detected in the mesenteric glands, albeit irregularly.

Intestinal Walls.—Thus far in the present investigations, recovery of virus from the intestines of normal (or paralyzed) mice has meant only its isolation from intestinal contents or feces. An attempt was now made to determine whether virus resides in the intestinal wall itself. That walls may contain virus has already been inferred (7, 12), but the question arises of whether they do so because of adherent virus-containing contents.

TABLE II

Virus in Mesenteric Glands

Experiment No.	Source mice				No. of glands used	Preparation of glands	Test mice injected intracerebrally		Remarks
	No.	Age	History	Virus in intestinal contents			No.	No. paralyzed	
		days							
1	2	43	Injected intracerebrally with intestinal contents of normal mice. Paralyzed 3 days	+	8	Suspension diluted 1:2 to 1:4	8	0	
2	3	57–63	Same. Paralyzed 3, 6, and 19 days	Not tested	9	Same	8	0	
3	3	60	Normal stock	+	20	"	8	0	
4	4	42	Same	+	20	1:2; 1:10	17	2	1:2 suspension of glands, 1 positive 1:10 suspension of glands, 1 positive
5	3	67	"	+	20	1:5	18	2	

In the experiments summarized in Table III, two methods of washing walls of small intestines were employed: in the one, the procedure of Theiler and Gard (7), namely, directing a stream of running tap water over the exposed mucosa for fully 2 minutes, was followed; in the other, the washing was done in a flask in four changes of saline solution, using 50 to 80 cc. of fluid for each washing and shaking the flask for a total period of 1 hour. The tissue was then dried gently with blotting paper and treated in the same manner as the contents: 5 to 10 per cent (by weight) suspensions in broth were centrifuged at 2,500 R.P.M. for 10 minutes; the supernate was passed through a Berke-

TABLE III

Virus in Contents and in Walls of Small Intestines of Normal and Paralyzed Mice

Experiment No.	Source mice: No.	Source mice: Age	Source mice: History	Test mice: 10^{-1} dilution* of contents injected intracerebrally: No.	Test mice: 10^{-1} dilution* of contents injected intracerebrally: No. paralyzed	Test mice: 10^{-1} dilution* of wall tissue injected intracerebrally: No.	Test mice: 10^{-1} dilution* of wall tissue injected intracerebrally: No. paralyzed	Duration of washing of walls	Remarks
		days							
1	2	43	Injected intracerebrally with intestinal virus of normal mice. Paralyzed 3 days	8	4	8	0	1 hr.	
2	3	30	Normal stock	8	8	8	3†	1 hr.	
3	3	30	Normal stock	10	10	10	3‡	2 min.	
4	3	30	Normal stock	8	7	8	7	1 hr.	Contents, 1:5 dilution
5	5	40	Normal stock	12	4	12	0	1 hr.	Both contents and walls, $10^{-2} + 10^{-3}$ dilution results combined
6	5	40	Normal stock	6	6	6	6	Unwashed, 10^{-1} dilution	
						6	3	Same, 10^{-2} dilution	
						6	0	" 10^{-3} "	
						6	5	Washed, 1 hr.	
						6	2	Same, 10^{-2} dilution	
						6	0	" 10^{-3} "	

* Unless otherwise stated.

† A later test for immunity (test dose, 10^{-1} dilution of contents which induced disease in all of seven normal mice but not in any of ten convalescents) was given to the five residual mice showing no paralysis; two proved resistant.

‡ In the same immunity test (as in Experiment 2), three of six residual mice showing no paralysis proved resistant.

feld V filter and 0.03 cc. of the filtrates, representing equivalent weights of intestinal walls or of contents, was injected intracerebrally into 30-day-old mice (Table III).

The results show that the experimental disease can be transmitted to mice more readily by means of intestinal contents than by washed walls.

A quantitative correlation of the virus contained in both materials is difficult to determine since it is not known precisely in just which structures it has its origin and consequently how much it is diluted, in preparations for inoculation, by inert matter that may be present in both contents and walls. Histological examination revealed that after 2-minute washing the mucosa and solitary lymph glands and other structures retained their normal appearance, although a considerable amount of contents still clung to the walls. After 1-hour washing in the manner described, the mucosa, except for the deepest layer, was washed away; the lymphatic glands and other structures remained whole and contents still adhered to the inner surface of the gut, although in traces, and then only in a spotty distribution. In view of the activity of contents in comparatively low dilution, it is not conceivable that the latter lends to the intestinal walls their activity. Taken in conjunction with the positive results obtained previously (7), the present ones point to the fact that the intestinal walls themselves harbor virus, although the particular place of the virus among their different structures has not as yet been fixed. The results of the following experiments on the effects of feeding croton oil might add support to the idea that the virus resides within the walls.

Experiments with Croton Oil.—The object of these tests was to eliminate the contents of the gut by drastic purgation and to determine whether shortly thereafter virus could be detected in the new contents.

It was found, after a number of trials, that the effective dosage of croton oil for the purpose in view was 0.5 cc. of a 10 per cent dilution of the oil in olive oil, given by gavage, under ether anesthesia. This amount was fed daily for from 1 to 5 days. The procedure was to feed enough croton oil to induce death of one-third to one-half the number of test mice, thus making certain that a drastic effect was secured, and to carry out a study of the contents of the small intestines of the survivors. The mice responded to the treatment by profuse semifluid, then fluid diarrhea, often showing blood-tinged stools which at times were associated with prolapse of the rectum, rapid and marked emaciation and cessation of activity. Histological examination of the intestines revealed necrosis with cellular, inflammatory reaction of the mucosa after one or two doses, and sloughing off of practically the entire membrane after three to five treatments. In certain ones the large masses of blood clot were formed in the lumen. In none was the central nervous system involved.

In spite of the action of croton oil as a drastic purgative, and its effect on the intestinal mucosa, the pale-yellowish, mucoid intestinal contents of mice a day after receiving one or more daily doses of croton oil still yielded virus.

It is of interest that of forty mice receiving croton oil, none exhibited

clinical signs of Theiler's disease, *i.e.*, the marked reaction of the intestinal lining failed to invite progression of the virus from the contents through the wall to the central nervous system.

Thus, it was first determined by means of positive transmissions obtained with their filtered feces, that a series of 35-day-old normal mice harbored intestinal virus. No food was given and the mice were kept isolated in separate cages having perforated platforms in order to prevent fecal accumulation and coprophagy. They were then given croton oil by gavage. 24 hours after one treatment, the pooled intestinal contents of two of the mice, in 10^{-1} dilution, induced paralysis in nine of ten intracerebrally inoculated animals; so also, after two daily treatments, the contents of three mice were active in nine of ten and, after four and five daily doses, encephalomyelitis developed in all of six and ten injected mice respectively. The limit of viral activity in the contents was at a 10^{-3} dilution—the same end-point of infectivity found in the untreated mice.

It would appear, therefore, that seeding or contamination of intestinal contents with virus proceeds apace from an origin which is not the contents as such, or food, but more likely is within the walls themselves. Moreover, the results in general are in good agreement with those of Theiler and Gard (7) who conclude, from a wholly different approach to the problem, that the persistence of intestinal virus is traceable to a reservoir within the mouse itself and not to some outside source.

To sum up the foregoing results, the virus is found in normal (and paralyzed) mice in their intestinal contents as well as walls, to a less degree in their mesenteric glands and in the central nervous system; in the latter tissue, however, only in mice having encephalomyelitis but not in normal animals.

The remainder of the present investigation was directed toward a study of certain other properties (11, 12) of the intestinal virus found in normal mice. The object was to note any difference in effect between it and Theiler's virus as it occurs in the spontaneous disease.

Susceptibility of Animals Other than Mice.—An attempt was made to convey clinical infection by means of intestinal virus obtained from normal mice, to guinea pigs, rabbits, and monkeys. These animals were injected intracerebrally with 0.15, 0.3, and 1 cc. respectively of 20 per cent suspensions of intestinal contents (about 200 mouse cerebral infective units) and intraperitoneally, at the same time, with 2.5, 5, and 5 cc. respectively of the material. Moreover, the monkeys received additional similar doses 6 days after the first administration. No signs of reaction, including fever, were seen in any of the animals during a period of observation of 40 days at least. Moreover, serum from two of the monkeys collected 30 days after the second dose of intestinal virus contained no neutralizing antibodies

against this active agent, as indicated by the results of the mouse intracerebral test. The insusceptibility of monkeys (1) and of rabbits (3) to Theiler's virus derived from spontaneous, or its experimentally passaged, infection has already been noted. In respect to resistance of certain animals other than mice, the intestinal virus therefore aligns itself with the strains of Theiler's virus.

Route of Inoculation of Intestinal Virus.—

Evidence is already at hand (11) to show that the intestinal virus which is uniformly active by the cerebral route, is not disease-producing when injected sub- or intracutaneously (abdomen, pads of feet), or applied to the scarified skin, or when fed by gavage or by cannibalism of infected mouse brain. On the other hand, Theiler's virus, as it occurs in the spontaneous or passaged disease, has been shown to be followed by clinical signs in small numbers of mice receiving it intranasally (five of twenty mice), subcutaneously (two of twenty), and in the plantar skin (two of ten); but intravenous, oral, intrasciatic, and intraperitoneal administration was ineffective (4). Gildemeister and Ahlfeld (3) obtained negative results by intranasal, intraperitoneal, and subcutaneous routes of inoculation. Theiler and Gard (7, 9), however, found that only with certain strains of this virus, designated as "unusually virulent" (*e.g.*, GD VII and FA), can paralysis be induced after intranasal and intraperitoneal administration.

Experiments were undertaken anew (11) to observe the effect of peripheral injection of intracerebrally active intestinal virus, derived from normal mice 32 to 40 days of age.

One group of mice was given 0.03 cc. of 1:5 dilution of unfiltered contents instilled intranasally and the same dose administered again 20 hours later; another received intraperitoneally 0.5 cc. of filtered material (diluted 1:10); and the third, unfiltered contents, diluted 1:2, applied to the scarified skin of the tail—a method that proves successful in experimental herpetic infection of mice for study of induced local and nervous signs (13). The ages of the mice in subgroups of five to ten animals, in which the active contents were instilled intranasally, were 14, 23, 35, and 90 days and of those receiving the material intraperitoneally and by cutaneous scarification, 15 and 20 days.

After such injections of virus originating from normal mice, neither characteristic encephalomyelitis, nor local lesions in those receiving active material in scarified skin, was induced in any of the animals, except in two of ten 14-day-old mice which received material by the nasal route. The latter two developed characteristic experimental encephalomyelitis. In the failure to bring about regularly, with the intestinal virus, clinically apparent infection by the peripheral routes mentioned, there is conformity with such strains of Theiler's virus, derived ultimately from the spontaneous disease, as are relatively low in invasiveness (7) after peripheral inoculation.

DISCUSSION AND SUMMARY

To the present time, including additional experiments still to be reported, over fifty trials have been made with intestinal contents collected from more than 200 normal mice of ages from 1 to 2 months. In all of the tests in which the contents of one, or pooled similar material of two to five mice were studied, a virus was recovered regularly, which produced encephalomyelitis indistinguishable from that of spontaneous Theiler's disease. The virus is probably present in an appreciable amount since the contents, although naturally containing a great deal of inert material, can be diluted from 1:100 to 1:1,000 and still be infectious. An interesting biological phenomenon is exhibited by (*a*) the almost universal presence of virus in apparently healthy mice of certain age; (*b*) its recovery from the intestinal tract and its uniform pathogenicity for the same species by intracerebral test; (*c*) the persistence of the carrier state for long periods of the host's life; and withal (*d*) the occurrence of spontaneous, clinically apparent disease in only one among thousands of animals. Examples similar, but only in certain respects, may be found in other fields of bacteriology: An instance is afforded by the observation of Sabin (14) relating to certain pleuropneumonia-like microorganisms. Although in some stocks of normal mice 80 per cent, in others, all animals may carry these microorganisms in their nasal mucosae or conjunctivae, the particular type of arthritis which can be brought about upon their inoculation into mice has not been observed to occur spontaneously. Again, practically all vertebrates, including man, harbor *Escherichia coli* in the intestinal tract; now and then local inflammations are set up by this bacterium, but its pathogenicity for animals is generally slight (15).

Another point of interest is that the active agent is not detectable in normal mice in their fetal stage to the age of 12 days. At the age of 20 days it becomes demonstrable, somewhat irregularly, however, and the irregularity is again observed in old mice (from 2 to 6 months of age or older). The loss of virus in older animals is correlated with the findings of Theiler and Gard (7) that with time carriers become free of virus. The onset of the carrier state at about the weaning period would imply at first glance contamination of the intestinal tract by food containing the virus—since feces are also infective—but this has yet to be definitely determined. At any rate, the persistence of the carrier state is not due to virus-contaminated food (7).

The intestinal virus derived from normal mice resembles in many of its properties Theiler's virus as it is recovered from spontaneous mouse encephalomyelitis or its passaged disease. The properties are similar

in respect to cross-active immunity (passive immunity, *i.e.*, serum-neutralization tests are not as yet available for want of a suitable method); to clinical reaction and pathological changes; to susceptibility of the mouse but not of other laboratory animals, including the monkey; and to other characteristics, such as size which is the same for both viruses, as determined by Theiler and Gard (9). The intestinal virus can, therefore, be regarded as a strain of Theiler's virus, and of the different strains hitherto described (7, 9) it belongs among those having a low invasive capacity after peripheral inoculation, *although the pathogenicity of the intestinal and the spontaneous active agent after intracerebral inoculation is the same.*

By the methods used in this investigation, normal mice of definite age reveal the presence of the virus uniformly and regularly in the intestinal contents. As Theiler and Gard (7) and we have shown, it is also recoverable from the walls, especially of the small intestines, but from what particular structure therein is still unknown. The contents, however, exhibit a higher percentage of incidence of paralysis than do the walls in the same dilution, respectively, used for animal inoculation. Furthermore, the active agent is found in the mesenteric glands but by the means here employed less regularly than in either contents or walls of the small intestine. No virus has as yet been recovered from the central nervous system of normal mice (7, 11, 12); it can be recovered from this tissue only of paralyzed animals. In relation to the presence of virus in intestines and mesenteric glands, no distinction can be made between normal and paralyzed mice; both harbor it to the same degree.

Moreover, in the large numbers of mice used for experimental purposes in our laboratory and injected intracerebrally with viruses other than Theiler's, or with a variety of non-virus materials, no increase in the incidence of spontaneous encephalomyelitis has been observed to follow such inoculations. So that the artificial production of injury to the brain does not in itself create the stimulus for centripetal progression of the peripherally located virus.

Whether the virus is produced in the mesenteric glands whence it progresses to the intestinal contents, or reversely, from contents to glands, is still unknown. Nor is it established as yet why in the rare animal, one among 4,000 or 5,000 (7, 12), the virus invades and then multiplies in the central nervous system, thus producing paralytic, spontaneous encephalomyelitis, although the virus is present in practically all of them.

CONCLUSIONS

Every experiment with the contents of one, or with those pooled from two to five of the normal stock of Rockefeller Institute strain of albino mice,

1 to 2 months of age, revealed the presence of a virus which, after intracerebral inoculation into normal mice, induced characteristic paralytic encephalomyelitis, indistinguishable from Theiler's disease. No difference was seen in this effect of intestinal contents deriving from animals paralyzed during the course of spontaneous encephalomyelitis and from normal mice. The influence of age on carriage of virus, as well as on the persistence of the carrier state, is discussed.

The present, as well as previous work has shown that the virus found in normal (or paralyzed) mice is similar to that of Theiler's disease in all of its properties thus far investigated; among the strains of the latter now at hand it can be classified with those having a low degree of invasiveness after peripheral inoculation.

The virus has thus far been recovered from intestinal contents, intestinal walls, and mesenteric glands but not from the central nervous system of normal mice; from these sites, as well as from the central nervous system, in paralyzed mice. In order of concentration of virus, the contents have more, the walls less, and the glands least.

BIBLIOGRAPHY

1. Theiler, M., *Science*, 1934, **80**, 122; *J. Exp. Med.*, 1937, **65**, 705.
2. Sabin, A. B., and Olitsky, P. K., *J. Exp. Med.*, 1938, **67**, 201.
3. Gildemeister, E., and Ahlfeld, I., *Centr. Bakt., 1. Abt., Orig.*, 1938, **142**, 144.
4. Iguchi, M., *Kitasato Arch. Exp. Med.*, 1939, **16**, 56.
5. Kligler, I. J., personal communication.
6. Poliomyelitis, International Committee for the Study of Infantile Paralysis, Baltimore, The Williams & Wilkins Co., 1932, 50–52.
7. Theiler, M., and Gard, S., *J. Exp. Med.*, 1940, **72**, 79.
8. Sabin, A. B., and Olitsky, P. K., *J. Immunol.*, 1936, **30**, 398.
9. Theiler, M., and Gard, S., *J. Exp. Med.*, 1940, **72**, 49.
10. For references, see Kling, C., Olin, G., Magnusson, J. H., and Gard, S., *Bull. Acad. méd.*, Paris, 1939, **121**, 826; Kramer, S. D., Gilliam, A. G., and Molner, J. G., *Pub. Health Rep., U. S. P. H. S.*, 1939, **54**, 1914; Levaditi, C., and Lépine, P., Les ultravirus des maladies humaines, Paris, Librairie Maloine, 1938, 595; Paul, J. R., Trask, J. D., and Culotta, C. S., *Science*, 1939, **90**, 258; Toomey, J. A., *Arch. Pediat.*, 1939, **56**, 693.
11. Olitsky, P. K., *Proc. Soc. Exp. Biol. and Med.*, 1939, **41**, 434.
12. Olitsky, P. K., *Proc. Soc. Exp. Biol. and Med.*, 1940, **43**, 296.
13. Andervont, H. B., *J. Infect. Dis.*, 1929, **45**, 366. Holmes, F. O., personal communication.
14. Sabin, A. B., *Proc. 3rd Internat. Cong. Microbiol.*, New York, 1940, 182.
15. Zinsser, H., and Bayne-Jones, S., A textbook of bacteriology, New York, D. Appleton-Century Co., Inc., 8th edition, 1939, 492.

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"SPREADING" PROPERTIES AND MUCOLYTIC ACTIVITY OF LEECH EXTRACTS

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In addition to their well known anticoagulating properties, leech extracts have the power to increase skin permeability.[1] Chemical studies from this laboratory have shown that the so-called spreading or "Reynals" factors, either from testicle,[2] or from the leech, were proteins.[3, 1] Recently, Chain and Duthrie[4] found that testicular extract would destroy the viscosity of the synovial fluid, and they have shown that this effect was due to the action of an enzyme on a polysaccharide. These authors suggested that this "mucinase" and the testicular spreading factor might be identical. If this is correct, one may expect leech extracts to have a high mucolytic activity, since their spreading power is 50 to 100 times greater than that of testicular extracts. In preliminary tests it was found that leech extract would destroy the viscosity of chicken tumor I extracts[5] with a remarkable speed, even at room temperature.

In the following experiments, the spreading power of various leech extracts was compared with their mucolytic activity, as measured by their action on chicken tumor I mucin.

Effect of Leech Extracts on Chicken Tumor I mucin. Previous work has shown that the spreading factor is located in the anterior part of the leech (head).[1] It is assumed that the substance is produced by the pharyngeal epithelium and plays a rôle in the process of feeding. On the other hand, a similar extract from the rest of the body is found to contain very little of the same factor.

In the present experiments, separated heads and bodies were extracted, in the usual way,[1] with 10 to 20 times their own weight of distilled water or phosphate buffer at pH 7.0, and centrifuged at 18,000 times gravity for 2

[1] Claude, A., *J. Exp. Med.*, 1937, **66**, 353.

[2] Duran-Reynals, F., *J. Exp. Med.*, 1929, **50**, 327; Hoffman, D. C., and Duran-Reynals, F., *Science*, 1930, **72**, 508; *J. Exp. Med.*, 1931, **53**, 387; McClean, D., *J. Path. and Bact.*, 1930, **33**, 1045.

[3] Claude, A., and Duran-Reynals, F., *J. Exp. Med.*, 1937, **65**, 661.

[4] Chain, E., and Duthrie, E. S., *Nature*, 1939, **144**, 977.

[5] Claude, A., *J. Exp. Med.*, 1935, **61**, 27.

hours. These extracts were used as the source of enzyme. The substrates for mucolytic activity were concentrated extracts of chicken tumor I desiccates,[5] purified by high speed centrifugation. The speed of the enzymatic reaction was studied at a temperature of 25°C.

Under these conditions the leech head extract, in the concentration of 1%, by volume, was found to have a powerful effect on the chicken tumor mucin, bringing the relative viscosity of the extract from 7.7 to 1.5 in 5 to 10 minutes. At that concentration, 30 minutes of contact with the leech factor brought the viscosity of the fluid close to that of water, the reduction in viscosity corresponding to 99.25% of the original value. Leech head extract, in a final concentration of 0.01% reduced the original viscosity by 73.6% whereas the leech body extract, in a final concentration of 1%, brought about a reduction in viscosity of 73.9%. This indicates that a 0.01% head extract and a 1% body extract have about equal strength, the former being then about 100 times more active than the latter.

The spreading power of the leech extracts was tested by the injection of 10-fold dilutions of the solutions, mixed with India ink, in the rabbit skin.[2,1] The head extract, at a dilution of 10^{-3}, gave an area of spread equal to 20.3 sq cm, as compared with 22.6 sq cm for the body extract, at 10^{-1} dilution. This would indicate that the head extract is about 100 times more active than the body extract. The above results are illustrated in Table I.

The close parallelism between the rate of action of the mucinase and the spreading power of different extracts is strong evidence that the two factors, in the leech, are identical.*

This view is also supported by the fact that bull testicular extract was about 100 times less active than the leech extract, as regards both spreading power and mucolytic activity.

Mechanism of Spread. No satisfactory explanation has been found to account for the phenomenon of spread.[1] The spread, considered as the result of the mucolytic activity of the spreading factor would assume the presence, in the skin, of a chemically suitable substrate for the mucinase to act upon. From the histochemical studies of Bensley[6] and Sylvén[7] it appears that normal or pathological tissues contain a viscid ground substance which, from its staining properties, resembles mucin.

In the present work freshly removed rabbit skin was passed through a

* The present work has been confined to the study of changes in viscosity under the effect of leech extracts assuming that the spreading phenomenon is brought about by similar changes in the skin without requiring necessarily complete hydrolysis of the substrate.

[6] Bensley, S. H., *Anat. Rec.*, 1934, **60**, 93.

[7] Sylvén, B., *Virchows Arch. Path. Anat.*, 1938, **303**, 280.

meat grinder and extracted by contact with 2 volumes of a 10% NaCl solution at 2°C for 24 hours. This extract was filtered through paper and the filtrate treated with chloroform, with occasional shaking, for another 24 hours. Denatured proteins were discarded by centrifugation. The clear solution was then dialyzed in cellophane bags until free of NaCl. A pro-

TABLE I

"Spreading" Property and Mucolytic Activity of Leech Extracts

Leech head extract					Leech body extract				
Spread in rabbit skin			Mucolytic activity		Spread in rabbit skin			Mucolytic activity	
Amt solids injected, g	Area of spread, cm^2	Ratio active spread to spread of control	Conc. leech extr. in mucin sol. (by vol.), %	Reduction viscosity after 30 min. at 25°C, %	Amt solids injected, g	Area of spread, cm^2	Ratio active spread to spread of control	Conc. leech extr. in mucin sol. (by vol.), %	Reduction viscosity after 30 min. at 25°C, %
3.6×10^{-3}	91.0	16.0	1.00	99.3	2.35×10^{-3}	66.9	11.7	1.0	73.9
3.6×10^{-4}	38.0	6.6	0.10	97.9	2.35×10^{-4}	22.6	4.0	0.1	35.2
3.6×10^{-5}	31.0	5.4	0.01	73.6	2.35×10^{-5}	8.0	1.4		
3.6×10^{-6}	20.3	3.2			2.35×10^{-6}	6.3	1.0		
3.6×10^{-7}	6.8	1.2			2.35×10^{-7}	7.0	1.0		
3.6×10^{-8}	8.0	1.4			2.35×10^{-8}	7.5	1.3		
3.6×10^{-9}	5.5	1.0			2.35×10^{-9}	6.2	1.0		

TABLE II

Mucolytic Activity of Leech Extract on Normal Rabbit Skin Mucoprotein

Mucoprotein solution			Leech extract (head)		Mucolytic activity			
			Amt added to mucoprotein solution		30 min at 25°C		30 hr at 25°C	
Tests	Solids in sol. mg per cc	Relative viscosity of sol.	By volume, %	Final conc. in mixture, %	Relative viscosity of mixtures	Reduction in viscosity %	Relative viscosity of mixtures	Reduction in viscosity %
I	0.28	1.44	0.74	0.003	1.12	72.7		
II	0.70	2.2	1.0	0.004	1.38	68.4		
"	0.70	2.2	0.1	0.0004	1.43	64.0		
III	0.70	2.2	1.0	0.004	1.46	61.7	1.32	73.4
IV	1.40	3.46	1.0	0.004	2.05	57.3	1.80	67.5

tein fraction, different from the mucoprotein, separated out on dialysis, and was discarded by filtration through paper. All the operations were conducted in the cold, to avoid the action of a mucolytic enzyme which might have been extracted from the skin, together with the mucoprotein.

The skin mucoprotein is readily soluble in water, giving clear, slightly yellow solutions. The biuret reaction is positive. Solutions containing 0.7 mg solids per cc gave a strongly positive orcinol test. Like chicken

tumor I mucin, it gives a stringy precipitate with neutral red. The behavior of the substance in acidic solution is noteworthy. Its solubility decreases progressively down to pH 4.0, giving a flocculent precipitate. At pH 4.0 there is a sudden change in the appearance of the precipitate which is then mucoid, and contracts. Below pH 4.0 the protein becomes more soluble but the precipitate retains its mucoid character. Below pH 2.0 the substance is completely soluble. On account of the physical change taking place at that point, it is uncertain whether pH 4.0 is the point of minimum solubility of the protein.†

A solution containing 1.4 mg skin mucoprotein per cc had a relative viscosity of 3.46 at 25°C. Addition of leech extract to a final concentration of 0.004% produced a sudden drop in the viscosity of the solution. As a result of 5 different tests, it appears that leech extracts, at the above concentration, and acting for 30 minutes at 25°C, will reduce the original viscosity of the solution by 68.4%. A contact of 30 hours at the same temperature will show but a slight additional effect, the total reduction in viscosity amounting to 70.4% of the original value. These results are in agreement with the observations of Meyer and coworkers,[8] who showed that only 69% of the synovial mucin was hydrolyzed by 45 hours' incubation with a pneumococcus enzyme, in contrast with 96% hydrolysis for the free polysaccharide, under the same conditions.

Summary and Conclusions. 1. Leech extracts contain a powerful mucolytic enzyme, as shown by its effect on the viscosity of chicken tumor I extracts. 2. Leech head extracts exhibit a mucolytic activity considerably greater than that of similar extracts obtained from the rest of the leech body. A comparable quantitative relationship is found to exist between head and body extracts when tested for another property, *e.g.*, their power to spread in the rabbit skin. 3. The parallelism in the strength of various extracts, as regards both mucolytic activity and spreading power, supports the view that the "mucinase" and the leech spreading factor may be identical. 4. A mucoprotein has been prepared from normal rabbit skin. 5. The viscosity of a skin mucoprotein solution is rapidly and considerably reduced by the action of leech extracts. 6. The effect of leech extracts on the skin mucoprotein *in vitro* suggests that their ability to spread through the skin may be due, at least in part, to their power to cause hydrolysis or depolymerisation of the same or a similar compound *in vivo*.

† The name "mucoprotein" refers especially to the physical properties of the material since the solution may contain different soluble components of the skin.

[8] Meyer, K., Hobby, G. L., Chaffee, E., and Dawson, M. H., *J. Exp. Med.*, 1940, **71**, 137.

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PROPERTIES OF THE CAUSATIVE AGENT OF A CHICKEN TUMOR

XIV. RELATION BETWEEN A TUMOR NUCLEOPROTEIN AND THE ACTIVE PRINCIPLE

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Repeated attempts have been made in the past to correlate the tumor-producing activity with the chemical constitution of chicken tumor extracts, and the ultraviolet absorption spectrum of these extracts has been used for that purpose on several occasions. Ordinary Berkefeld filtrates of Chicken Tumor I were found to possess a marked absorption in the ultraviolet region, at $\simeq \lambda 2600$, and a weaker range of absorption at $\lambda 4100$ (1). Extracts purified by adsorption and dialysis retained their tumor-producing property, and still absorbed ultraviolet light, with the suggestion of a maximum at $\simeq \lambda 2550$ (1). Recently, a fraction which carried practically all the initial tumor-producing activity was separated from chicken tumor extracts by means of differential centrifugation at high speed. The material, purified in this manner, was found to exhibit an ultraviolet absorption spectrum strikingly similar to that of the tumor extract purified by adsorption and dialysis, with indication of a maximum of absorption at $\lambda 2575$ (2). The fact that active tumor fractions, although prepared by entirely different methods, possess the same absorbing power for ultraviolet light led us to reinvestigate the relation between the absorbing elements and the tumor principle.

Materials and Methods

Preparation of Tumor Extracts.—The mode of preparation of purified and highly active tumor material has been briefly described in previous notes (3).

The tissue from selected tumors, frozen at −80°C. and stored at that temperature for 3 to 15 days, was used in these experiments. The frozen tissue was ground with sterile sand and extracted with a 0.005 M phosphate buffer solution at pH 7.0. This tissue suspension was centrifuged for 30 minutes at 2400 times gravity, in an angle

centrifuge.[1] The deposited tissue was extracted once more in exactly the same manner and the two supernatant fluids were combined. This material will be referred to as the tumor extract. The total volume of buffer solution used for the double extraction corresponded to 15 times the weight of the tumor tissue. The average solid material of this tumor extract was about 3.4 mg. per cc., and the specific viscosity of the fluid was approximately 3.2 times that of water.

Purification of the Active Fraction in the High Speed Centrifuge.—The tumor extract was centrifuged under a force of 17,000 to 18,000 times that of gravity for a period of 2 to 3 hours, depending on the viscosity of the fluids.[2] The supernatant fluid was discarded and the sediment was resuspended in a small volume of phosphate buffer solution. Coarse particles were removed from the suspension by a short run of 3 minutes at high speed. The coarse sediment was resuspended in buffer solution, redeposited by a short run of 3 minutes, and discarded. The supernatant fluids from the different short runs, which were found to contain the active principle, were combined and saved for further purification in the centrifuge.

The entire process, consisting in a long run followed by two or three successive short runs of 3 minutes at high speed, was repeated twice more. In the end, the purified tumor agent was taken up in 0.005 M phosphate buffer solution at pH 7.0, the final volume being equal to one-tenth that of the original extract. This last suspension will be referred to as the "purified fraction." During the experiment, the temperature of the material was maintained near 0°C., except for the first long run at high speed, when it attained 12–16°C.

Tests of Activity.—Chicken tumor extracts gradually lose their activity *in vitro* and the tumor agent appears to be even less stable in the purified form. For this reason, efforts were made to test the purified fraction the day of its preparation, extraction of the frozen tissue and treatment of the extract in the high speed centrifuge requiring 12 to 14 hours.

The tumor-producing power of the purified material was determined by injecting 0.4 cc. of the test solutions into the skin of adult Plymouth Rock hens. Dilutions of the stock solutions were made with a 0.005 M phosphate buffer solution at pH 7.0, containing 2 per cent rabbit serum. New 1 cc. pipettes were used for the first three dilutions. The results were recorded by measuring the size of the tumors which were present, not later than 18 days after inoculation.

Determination of Ultraviolet Absorbing Power.—Measurements of the absorbing power of the solution for ultraviolet light were carried out with a Hilger sector-photometer as previously described (1). In recording the curve, the extinction coefficients were calculated from the relation $I = I \times 10^{-\epsilon c}$ where c was the concentration arbitrarily expressed in grams per 10 cc. In a few experiments, the source of light was the continuous spectrum of a hydrogen discharge tube. Absorption measurements were carried on the freshly prepared material at the same time at which inoculation tests were performed.

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[1] International Equipment Co., Boston, type S.B., size 1 centrifuge in conjunction with conical head No. 283.

[2] The device used in these experiments was the multispeed attachment and No. 295 head provided by the International Equipment Co., Boston, for their type S.B., size 1 centrifuge.

EXPERIMENTAL

Tumor-Producing Activity of the Purified Tumor Fraction and Ultraviolet Absorption Spectrum

The results obtained in the following experiment are typical for the purified fraction prepared according to the method described above.

Tumor-Producing Power of the Purified Fraction.—The results of inoculation, based on four tests at each dilution, are given in Table I. It shows that the purified fraction was fully active when brought to a dilution of 6.5×10^{-10}, as calculated from the weight of the original tumor tissue. Inoculation of 4.0×10^{-13} gm. of the purified substance was sufficient to produce well developed tumors 18 days after injection. In this experiment,

TABLE I

Tumor-Producing Power of the Purified Fraction

Results of Activity Tests 18 Days after Injection

Dilution	Tumor extract (control)			Purified fraction		
	Solids injected	Takes	Tumor size	Solids injected	Takes	Tumor size
	gm.	*per cent*	*cm.*	*gm.*	*per cent*	*cm.*
6.5×10^{-5}	1.7×10^{-5}	100	2.3×1.8	4.0×10^{-8}	100	2.5×1.9
6.5×10^{-6}	1.7×10^{-6}	100	2.5×1.6	4.0×10^{-9}	100	1.8×1.6
6.5×10^{-7}	1.7×10^{-7}	100	1.9×1.5	4.0×10^{-10}	100	1.9×1.6
6.5×10^{-8}	1.7×10^{-8}	75	1.5×1.2	4.0×10^{-11}	100	2.1×1.8
6.5×10^{-9}	1.7×10^{-9}	100	1.4×1.1	4.0×10^{-12}	100	1.8×1.4
6.5×10^{-10}	1.7×10^{-10}	0	—	4.0×10^{-13}	75	1.5×1.2

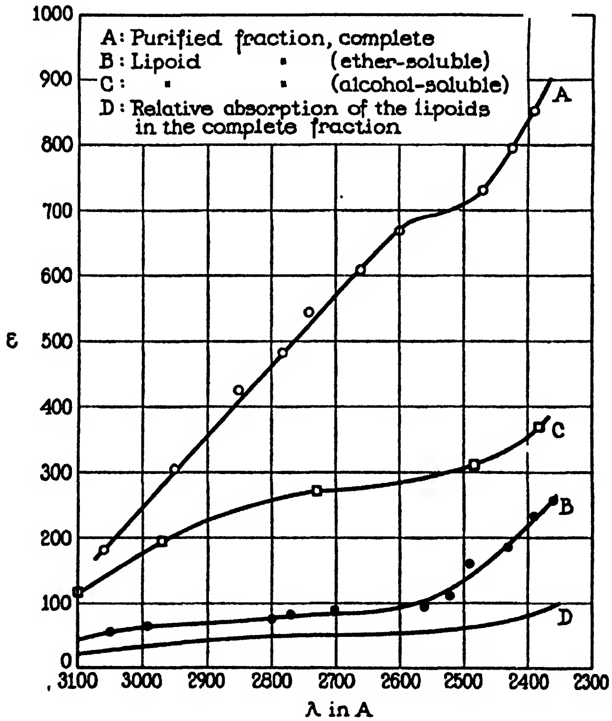
as can be seen from Table I, the purified fraction exhibited a tumor-producing power at least ten times greater than that of the unfractionated extract, kept as control at 4°C. This relative enhancement of activity shown by the material purified by centrifugation was observed consistently.

Absorption Spectrum of the Purified Fraction.—The ultraviolet absorption spectrum of the above material was determined. The preparation was a 0.017 per cent solution of the purified substance in 0.005 M phosphate buffer at pH 7.2.[3] As shown by the results of activity tests, this solution contained at least 4.2×10^{8} active doses per cc. Its ultraviolet absorption spectrum is given in Text-fig. 1, curve A, which shows a maximum at λ2575. This curve is quite similar to that obtained for nucleoproteins (4).

When the present results are compared with previous data (1, 2) it is

[3] The hydrogen ion concentration of all solutions studied was determined by means of a glass electrode.

apparent that improved methods of purification are reflected in a marked increase in the ultraviolet absorbing power of the purified fraction, and this is paralleled by an increase in tumor-producing activity. At λ2575, the extinction coefficient of the fraction purified by adsorption and dialysis and subsequently concentrated, was $\epsilon = 135$ (1). Material purified by high speed centrifugation, but with no more than 10 to 20 per cent of the



TEXT-FIG. 1. Absorption spectra of the purified tumor fraction and of its lipoid constituents.

activity of the original extract, showed, for the same wave length, an extinction coefficient $\epsilon = 525$ (2). In the present work the extinction coefficient of highly active preparation was found to be consistently in the neighborhood of $\epsilon = 700$ for the wave length λ2575. In each case, however, the absorption curve is essentially the same, with the maximum at practically the same wave length, λ2575. From these observations, it seems that a correlation may exist between the absorbing power and the activity of the solution.

Absorbing Power of the Lipoid Constituents of the Purified Fraction.—Previous studies have shown that part of the purified fraction was lipoid in

nature, the other portion being represented, to a large extent, by a nucleoprotein (5).

Separation of the lipoids from the purified fraction was accomplished by extracting the dried substance with ether and alcohol. The freshly purified tumor fraction was frozen at −80°C. and desiccated *in vacuo* in the frozen state. Extraction was performed at room temperature by leaving the dry substance in contact with the solvent for successive periods of 2 hours, with occasional stirring. The material was extracted three times with ether, and then three times with absolute alcohol. In terms of dry weight, the ether-soluble and the alcohol-soluble portions represented 15.4 and 14.3 per cent of the original material, respectively. No purification of these lipoids was attempted, each fraction being probably a mixture of several components. For the spectrographic measurements, the ether and alcohol fractions were dissolved in heptane and examined separately.

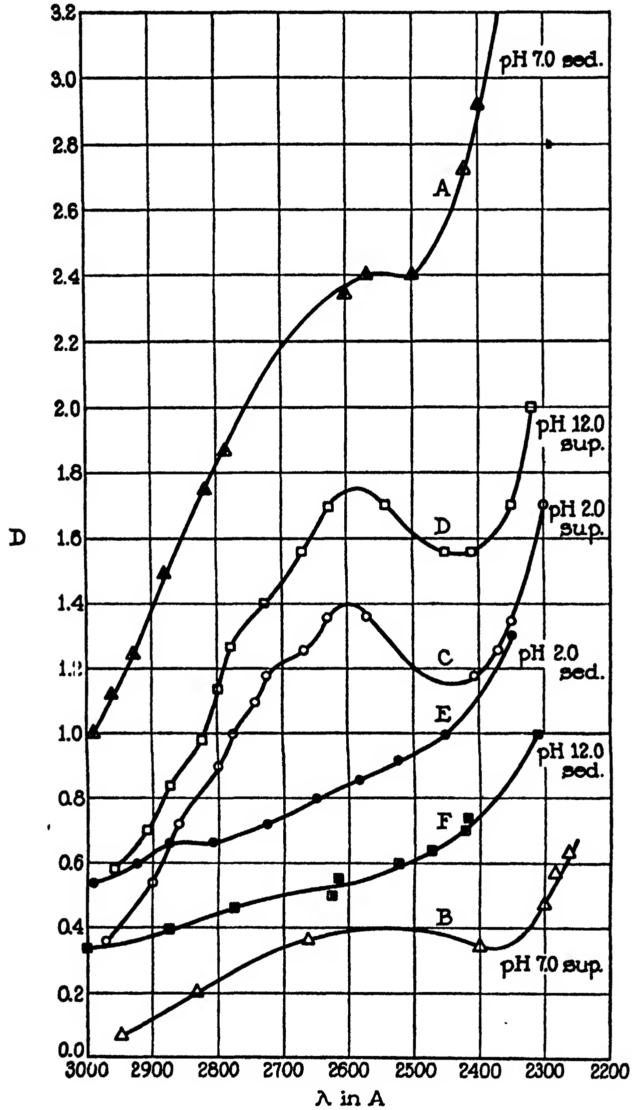
The results are given in Text-fig. 1. Curve B represents the absorption spectrum of the ether fraction, curve C that of the alcohol fraction. It shows that both materials have some absorbing power in the ultraviolet region, but no apparent maximum. At λ2575, the combined lipoids account for about 8.5 per cent of the total absorbing power of the complete fraction, as can be seen from Text-fig. 1, curve D, where the ordinate represents the sum of the extinction coefficients of the two lipoids multiplied by the relative concentration of the latter as it occurs in the complete purified fraction. It is apparent that for λ2900 to λ2600 most of the absorbing power of the purified fraction is a property of the nucleoprotein component of the material (4).

Effect of Acid and Alkali on the Purified Tumor Fraction

Lewis and Michaels have shown that ordinary chicken tumor extracts, adjusted to various hydrogen ion concentrations by means of appropriate buffers, retained their tumor-producing activity for at least 30 minutes in the range between pH 4.0 and pH 12.0, whereas inactivation occurred immediately at, or beyond pH 3.6 and pH 12.5 (6). The results which follow indicate that these findings apply also to the tumor agent in the purified form.

The solubility of the purified tumor fraction was investigated by mixing the stock preparation, in neutral water, with an equal volume of 0.1 M buffer solutions of the proper pH, the final concentration of the substance in the mixture being 0.14 mg. per cc. Under these conditions, the purified fraction forms stable colloidal solutions between pH 7.0 and pH 11.0. Between pH 11.5 and 13, the solution becomes rapidly more transparent. On the acid side, the substance is very sensitive to an increase in hydrogen

ion concentration. Aggregation begins to take place already at pH 6.6, as shown by an increase in the opalescence of the solution. Between pH



TEXT-FIG. 2. Absorption spectra of the purified fraction after treatment with acid or alkali. The ordinates D represent the absorption coefficients in arbitrary units.

4.8 and 2.4 the substance is practically insoluble, the point of minimum solubility being found in the neighborhood of pH 3.5. In solutions more acid than pH 2.4 the material is again more soluble, giving nearly clear solutions at pH 1.0. An interesting correlation appears to exist between

the points of rapid inactivation of the tumor agent and the stability of the purified substance in acid and alkali. Immediate inactivation occurs, on the acid side, at the point of minimum solubility of the purified tumor fraction, *i.e.*, pH 3.5, and on the alkaline side in the region of pH 12.0, where a disintegration of the substance seems to take place. At room temperature, the purified fraction retains its tumor-producing power for at least one hour at pH 4.0 or pH 11 (7). These observations led us to investigate the possible chemical changes produced in the solution, especially at those pH's at which the tumor agent is rapidly inactivated.

To 2 cc. of purified fraction, containing about 1.2 mg. substance per cc., were added 3 cc. of 0.02 N HCl or 3 cc. 0.01 N NaOH solutions, the final pH's being 2.0 and 12.0, respectively. The solutions were neutralized by means of 0.1 N NaOH or 0.1 N HCl, after a period of 1 hour. A sample of the purified fraction was diluted with 0.005 M phosphate buffer and kept at pH 7.0, as control.

In order to demonstrate the possible formation of split products during the treatment with acid or alkali, the neutralized solutions were submitted to high speed centrifugation, at about 18,000 times gravity, for 1 to 2 hours. The sediments were resuspended in neutral buffer and the volume adjusted to that of the supernatant fluids. Both the supernatant fluids and sediments were then examined for their power to absorb ultraviolet light.

The results are shown in Text-fig. 2. Curve A represents the absorption spectrum of the sediment recovered from the sample kept at pH 7.0. It is practically identical with the absorption spectrum of the standard tumor fraction, as illustrated in Text-fig. 1, curve A. Curve B represents the absorption of the corresponding supernatant fluid, showing that absorbing elements were practically absent from the solution. On the other hand, the results were quite different with the samples treated at pH 2 and pH 12. In this case, the supernatant fluids were found to contain large quantities of substances absorbing especially in the region of $\lambda 2600$ (Text-fig. 2, curves C and D), whereas the corresponding sediments, brought back to the original volume, exhibited an absorbing power considerably reduced (see Text-fig. 2, curves E and F). Thus, treatment of the purified fraction at pH 2 or pH 12 liberates certain absorbing constitutents which are no longer sedimentable under a force of 18,000 times gravity for 2 hours. The character of the absotption curve suggests that the substance set free at pH 2 and pH 12 is probably nucleic acid. This is supported by the fact that pH 2 and pH 12 supernatant fluids gave strongly positive tests for pentoses.

Effect of Ultraviolet Light on the Purified Fraction

The susceptibility of the chicken tumor agent to ultraviolet light was demonstrated by a number of workers who endeavored to determine the

relative amount of energy required for complete inactivation (8). Using monochromatic light and plotting the absolute amount of energy against the corresponding wave length, Sturm, Gates, and Murphy were able to establish a curve of inactivation which showed that the most effective region was in the neighborhood of λ2600 (9).

In the following experiments, the purified fraction was exposed to ultraviolet radiations and the absorption spectrum was determined during the course of irradiation to register any change which might take place under these conditions.

The purified fraction was kept in the quartz cell of the spectrograph and exposed to the condensed spark of tungsten steel electrodes, using 6 amperes at 110 volts for periods varying from 5 minutes to 5 hours, the material being left in place for the absorption measurements. During the treatment, the temperature did not rise above 26°C. A control for activity was kept at the same temperature during the experiment.

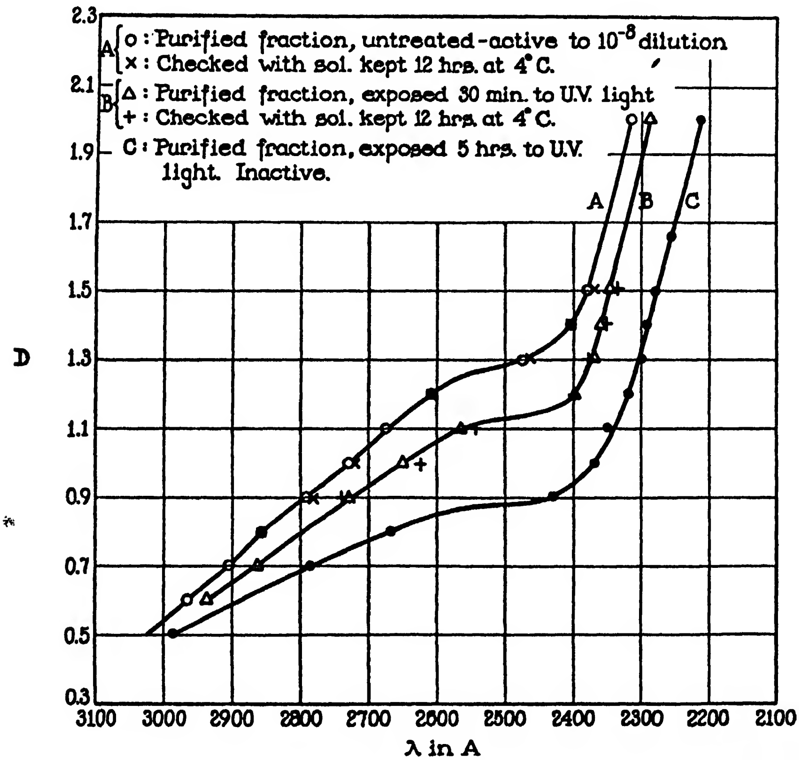
A summary of the results is given in Text-fig. 3. Curve A represents the absorption spectrum of the purified fraction, which contained 0.14 mg. dry substance per cc. and proved active at 10^{-8} dilution. The absorption spectrum of the same solution was redetermined after being kept for about 12 hours at 4°C. and was found unchanged. Curve B represents the absorption spectrum of the purified fraction after 30 minutes exposure to ultraviolet light. The absorbing power of the solution was appreciably decreased, especially in the region between λ2700 and λ2400. At λ2550, the original absorbing power of the solution was reduced by about 12 per cent. Inoculation tests showed that the tumor-producing activity had been completely abolished by this 30 minute irradiation. The material was kept at ice box temperature and the curve was redetermined 12 hours later. No further changes in the curve occurred. Continuous exposure of the purified material to ultraviolet light from a mercury arc, using 6 amperes at 110 volts, at 20 cm. distance for 5 hours, reduced the absorbing power of the purified fraction to about 69 per cent of the original value at λ2550 (see curve C).

Effect of Heat on the Purified Tumor Fraction

Rous and Murphy found that the tumor agent which is present in extracts of tumor desiccates was completely inactivated by heating at 55°C. for 15 minutes, but the tumor-producing power of the material was still demonstrated after heating at 50°C. for the same length of time (10). The following tests indicate that the same temperature range is also effective in bringing about rapid inactivation of the purified tumor agent. Since

mere dilution of the purified fraction with water or buffer solutions often causes an appreciable reduction of the tumor-producing power, relatively concentrated preparations and large volumes of material were used in the heating experiments.

Freshly prepared solutions, containing 1.84×10^{-4} gm. of the purified fraction per cc. in 0.005 M phosphate buffer at pH 7.0, were heated, in 8 cc. lots, to 50° or 65°C.



TEXT-FIG. 3. Effect of ultraviolet radiations on the absorption spectrum of the chicken tumor fraction. The ordinates D express the absorption coefficients in arbitrary units.

for exactly 30 minutes. The samples were enclosed in sealed tubes which were immersed in water baths maintained at the correct temperature. After 30 minutes, the solutions were cooled under running water and stored at ice box temperature until tested. The control solution was kept under the same conditions, but at 4°C., during the experiment. The results of the inoculation tests are given in Table II. It shows that heating the purified fraction 30 minutes at 50°C. had destroyed about 99 per cent of its tumor-producing activity. Heating 30 minutes at 65°C. abolished completely the tumor-producing power of the material.

No important physical changes were produced upon heating at these temperatures, except for a slight decrease in the opalescence of the solu-

tion. The ultraviolet absorption spectrum of the heated solution was determined as usual, and compared with that of the non-heated control. The results are given in Text-fig. 4, which shows that heating the purified fraction to 50° or 65°C. did not modify appreciably its absorption spectrum, the slight differences which are noted being within the limits of experimental error. These observations are in agreement with previous observations (1). Curve A represents the absorption spectrum of the control solution, which proved active at 10^{-8} dilution. Curve B represents the absorption of the solution heated 30 minutes at 50°C., and curve C corresponds to the solution heated 30 minutes at 65°C. As seen from those curves, inactivation of the tumor agent by heat does not affect the ultra-

TABLE II

Effect of Heat on the Tumor-Producing Power of the Purified Fraction

Measurements 18 Days after Inoculation

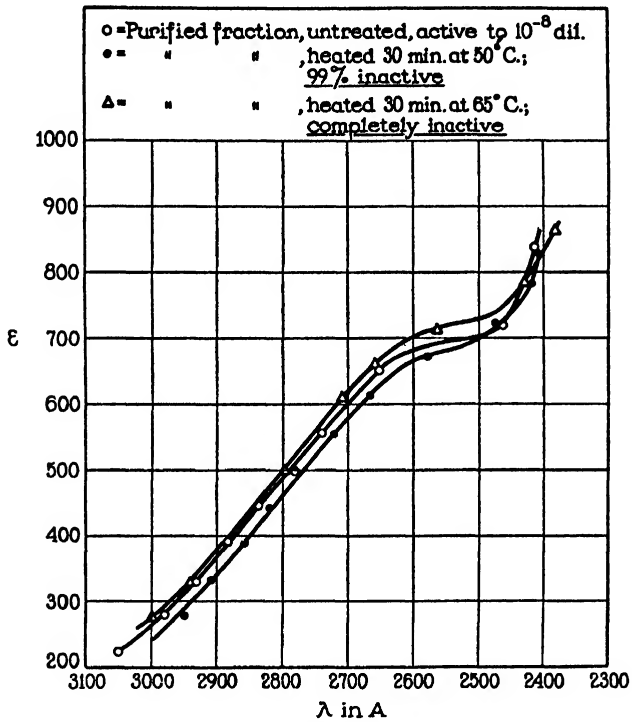
Dilution	Control		30 min. at 50°C.		Control		30 min. at 65°C.	
	Average size of tumors	Takes	Average size of tumors	Takes	Average size of tumors	Takes	Average size of tumors	Takes
	cm.	*per cent*	*cm.*	*per cent*	*cm.*	*per cent*	*cm.*	*per cent*
6×10^{-3}	2.9 × 2.5	100	2.0 × 1.6	100	2.3 × 2.3	100	—	0
6×10^{-4}	2.6 × 2.5	100	1.6 × 1.6	100	2.4 × 2.3	100	—	0
6×10^{-5}	2.0 × 1.8	100	1.2 × 1.1	100	2.0 × 1.0	100	—	0
6×10^{-6}	1.9 × 1.2	100	1.2 × 0.9	100	1.7 × 1.1	100	—	0
6×10^{-7}	1.8 × 1.3	100	—	0	1.2 × 0.8	100	—	0
6×10^{-8}	1.2 × 1.1	100	—	0	1.0 × 0.8	100	—	0

violet absorption spectrum of the purified fraction. Since the specific absorbing power of the purified fraction can be ascribed to the presence of a nucleoprotein, these results could have been expected, because the main absorbing component—nucleic acid—is known to be quite stable at this temperature range. However, the failure to detect a change in the total absorbing power of the solution upon heating did not exclude the possibility of a disruption of the nucleoprotein, an action which may not necessarily affect the structure of the chromophoric group responsible for the ultraviolet absorption power. In order to investigate an effect of this kind, an attempt was made to separate the possible products of dissociation by submitting the heated material to high speed centrifugation.

In these experiments, preparation of the purified material, heating of the solution, and subsequent separation in the high speed centrifuge, was performed the same day in order to avoid possible spontaneous deterioration of the material on standing. 8 cc.

lots of the freshly purified substance were heated for 30 minutes at 50° and 65°C. respectively, a control sample being kept on ice during the same length of time. Immediately after this treatment, the solutions were cooled to 0°C. and then centrifugalized for 2 hours at about 18,000 times gravity. The supernatant fluids were removed and centrifuged once more, at the same speed, for one hour. These twice centrifuged solutions were then examined for their power to absorb ultraviolet light.

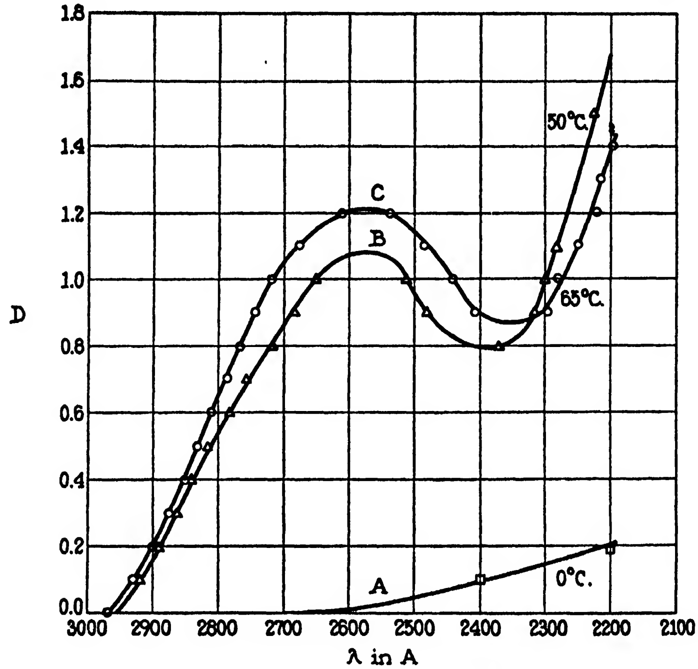
The results are recorded in Text-fig. 5. Curve A represents the absorption spectrum of the supernate derived from the control sample kept at 0°C.



TEXT-FIG. 4. Effect of heat on the ultraviolet absorption spectrum of the purified fraction.

As could have been expected, this solution was completely transparent and contained probably nothing but the phosphate buffer. On the other hand, solutions obtained from the heated materials exhibited a marked absorbing power, with a characteristic maximum at λ2575. Curves B and C correspond to the supernates obtained from the materials heated for 30 minutes at 50°C. and 65°C. respectively. As shown in Table III, the 50° and 65°C. supernates gave negative biuret tests but strongly positive tests for pentoses (Biall's test). The sediments from the first high speed centrifugation were washed once in buffer and resuspended in a volume of

buffer equal to that of the original solution. As shown in Table III, the "heated" sediments retained the protein components of the purified fraction, but lost most of the substance which, in the untreated sample, gave



TEXT-FIG. 5. Effect of heat on the purified tumor fraction; liberation of "nucleic acid" and its separation in the high speed centrifuge. The ordinates D express the absorption coefficient in arbitrary units.

TABLE III

Color Tests for Proteins and Pentoses, on Fractions Derived from Purified Tumor Material, Treated at Various Temperatures

Temperatures at which purified material was treated	Biuret		Biall's	
	Supernate	Sediment	Supernate	Sediment
°C.				
0	−	+	−	++
50	−	+	+±	±
65	−	+	++	±

the characteristic reaction of pentoses. The above observations indicate that heating the tumor fraction at 50°C. for 30 minutes, a treatment which destroys 99 per cent of the tumor-producing activity, causes the separation of an unsedimentable, ultraviolet-absorbing substance. Heating at 65°C.

for the same length of time, a treatment which destroys all the tumor-producing power of the material, causes more of the absorbing element to pass into solution. The characteristic absorption spectrum and color tests suggest that the substance of low molecular weight liberated by heat is nucleic acid.

DISCUSSION

The experiments reported in this paper indicate that the active chicken tumor fraction, isolated by differential centrifugation at high speed, presents a characteristic absorption in the ultraviolet, a maximum being found in the region of $\simeq \lambda 2575$. The findings are in agreement with previous work in which purified fractions obtained by other methods had been found to absorb ultraviolet light in the same manner. Absorption in that region is probably due, to a large extent, to the presence of purine and pyrimidine bases, since as much as 10 to 15 per cent of the material can be isolated in the form of nucleic acid. In the active fraction, the latter is apparently a constituent of a nucleoprotein.

The present observations establish the fact that agents which tend to inactivate the tumor principle will at the same time cause fundamental changes in the constitution of the nucleoprotein. Inactivation of the tumor agent by acid or alkali is accompanied by decomposition of the nucleoprotein into an insoluble protein and free nucleic acid of low molecular weight. Inactivation of the tumor agent by heat likewise corresponds to partial decomposition of the nucleoprotein and the release of nucleic acid into solution. On the other hand, inactivation of the agent by means of ultraviolet light is accompanied by a general decrease in the absorbing power of the purified fraction, this effect resulting probably from important changes brought about in the structure of the nucleoprotein molecule. Heyroth and Loufbourow (11) and Caspersson (12) have found that the ultraviolet absorbing power of nucleic acid is considerably reduced when this substance is submitted to ultraviolet irradiation for some time, the general shape of the curve, however, remaining the same. From Caspersson's results it is apparent that the rate in the decrease of absorption slows down with time of irradiation, and that an equilibrium is probably reached. From our results mentioned above, ultraviolet irradiation is found to affect the purified fraction in a similar manner. The change brought about in the nucleic acid molecule by irradiation has not yet been elucidated. It involves probably saturation or rearrangement of the double bonds in the purine and pyrimidine bases, a change which would influence considerably the property of the substance.

These changes which affect at the same time the tumor agent and the nucleoprotein might be coincidental, and the experiments do not prove that the nucleoprotein found in the purified fraction is necessarily an integral part of the tumor agent. In favor of the view, however, that nucleic acid is an essential constituent of the tumor principle is the fact that the wave length interval in the ultraviolet, which is the most effective in inactivating the tumor agent, coincides with the region of maximum absorption for nucleic acid. In this relation it may be recalled that the inactivation curve of Sturm, Gates, and Murphy (9) is practically reciprocal to the ultraviolet absorption curve of nucleic acid.

One may suppose that the mass of the purified fraction is composed mainly of inert matter, perhaps normal constituents of cells, whereas the part corresponding to the tumor agent is too small to be detected by the analytical methods which we have used. In this case the evidence would still point to a nucleoprotein as a probable constituent of the tumor principle.[4]

SUMMARY

1. The tumor-producing fraction, isolated from Chicken Tumor I by means of differential centrifugation at high speed, has been investigated as regards its power to absorb ultraviolet light. A characteristic absorption spectrum was found, with a maximum at λ2575. The absorbing power of the material in that region was largely due to the presence of nucleic acid, or of a closely related compound.

2. Inactivation of the purified tumor fraction with ultraviolet light depressed the absorbing power of the material, especially in the region of 2600–2500Å. These changes were those which nucleic acid would present under the same conditions.

3. Inactivation of the tumor agent with acid or alkali was accompanied by decomposition of the tumor nucleoprotein and passage of free nucleic acid into solution.

4. Partial or complete inactivation of the tumor agent by heat, at 50° or 65°C., was attended by liberation of nucleic acid of low molecular weight.

[4] Wyckoff (13) observed the presence of large amounts of unsedimentable material in preparations of purified bacteriophage (Northrop, 14), kept at pH 10 for 3 days. At that pH, bacteriophage solutions lose their activity rapidly. Mosaic virus protein decomposes progressively when the pH is raised above 9.0, with the appearance of unsedimentable material in the solution (15). Since these preparations have been shown to contain nucleic acid, it is probable that in the cases quoted the unsedimentable and ultraviolet-absorbing material was largely nucleic acid.

5. The parallelism between tumor-producing activity and the integrity of the tumor ribonucleoprotein suggests that the nucleoprotein may be an essential part of the active principle.

BIBLIOGRAPHY

1. Claude, A., and Rothen, A., *Am. J. Cancer*, 1936, **26**, 344.
2. Claude, A., *Am. J. Cancer*, 1937, **30**, 742; *Science*, 1938, **87**, 467; 1939, **90**, 213.
3. Claude, A., *Am. J. Cancer*, 1937, **30**, 742; *J. Exp. Med.*, 1937, **66**, 59; *Science*, 1938, **87**, 467.
4. Marchlewski, L., and Warzuchowska, J., *Bull. internat. Acad. polon. sc. et lett., Cl. sc. math. et nat.*, series A, 1928, 471.
5. Claude, A., *Science*, 1938, **87**, 467; 1939, **90**, 213.
6. Lewis, M. R., and Michaelis, L., *Bull. Johns Hopkins Hosp.*, 1928, **43**, 92.
7. Claude, A., *Am. J. Cancer*, 1937, **30**, 742; also unpublished experiments.
8. Rous, P., *J. Exp. Med.*, 1913, **18**, 416. Baker, S. L., and Peacock, P. R., *Brit. J. Exp. Path.*, 1926, **7**, 310. Illingworth, C. F. W., and Alexander, G. L., *J. Path. and Bact.*, 1927, **30**, 365.
9. Sturm, E., Gates, F. L., and Murphy, Jas. B., *J. Exp. Med.*, 1932, **55**, 441.
10. Rous, P., and Murphy, Jas. B., *J. Am. Med. Assn.*, 1912, **58**, 1938.
11. Heyroth, F. F., and Loufbourow, J. R., *J. Am. Chem. Soc.*, 1931, **53**, 3441.
12. Caspersson, T., *Skand. Arch. Physiol.*, 1936, **73**, suppl. 8.
13. Wyckoff, R. W. G., *J. Gen. Physiol.*, 1937-38, **21**, 367.
14. Northrop, J. H., *J. Gen. Physiol.*, 1937-38, **21**, 335.
15. Wyckoff, R. W. G., *J. Biol. Chem.*, 1937-38, **122**, 239.

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A DOG TEST FOR MEASURING THE IMMUNIZING POTENCY OF ANTIRABIES VACCINES

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Plate 35

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In a previous paper we described a mouse test for determining quantitatively the immunizing potency of antirabies vaccines (1). That test showed commercial phenolized vaccines for the most part to be incapable of rendering mice immune to a subsequent injection of rabies virus when employed in doses comparable to those for dogs or man with respect to body weight. Commercial chloroformized vaccines from two firms, on the other hand, showed definite immunizing capacity provided two to five times the comparative dose was given intraperitoneally rather than subcutaneously.

These findings have now been checked in dogs by determining whether canine vaccines which fail to immunize mice, likewise fail to immunize dogs, and conversely, whether vaccines plus procedures which successfully immunize mice, will likewise immunize dogs. The results of these tests thus far parallel those of the mouse tests (2, 3) and are described here in detail.

Technique for Testing Single Injection Antirabies Vaccines in Dogs

According to reports of previous workers, rabies vaccines prove capable of inducing a well defined immunity in animals only if they are administered prophylactically, that is, prior to a test injection of virus (5). Moreover, no method of testing thus far reported, save the one in mice (1), has yielded quantitative data on the amount of immunity obtained per unit of vaccine. The following test in dogs has been developed after the pattern of the mouse test to provide a quantitative result under conditions as nearly natural as possible.

Beagle dogs are used because of their small size and quiet disposition. Animals of the same age, that is, 4 to 6 months, are chosen because age factors are known to influence markedly the susceptibility and immunizability of mice to rabies (4), and they may play an equally important rôle with dogs. The young animals are brought to the premises usually in batches of about thirty-five, placed in quarantine rooms, wormed,

and given 10 to 15 cc. of distemper immune serum, followed 3 days later by one injection of non-virulent distemper vaccine.

About 7 days after their arrival, the animals are injected with the antirabies vaccines according to the requirements of the experiment. Five to fifteen animals remain unvaccinated, as controls.

3 weeks later, all are tested for immunity by an injection into the neck muscles of each side with 0.25 cc. of virus properly diluted. Dilutions are employed which are calculated to contain approximately 1, 10, or 100 lethal doses of virus (see Table I). The injected animals are transferred to single cages and observed 2 to 5 months. Those found prostrate or dead are autopsied, their brains removed, and a portion of the latter is inoculated intracerebrally into mice for identification of the virus. Immunity is measured in terms of number of lethal doses resisted by at least 50 per cent of the dogs.

The test virus, strain 15811, was obtained in 1937 from a rabid street dog. The virus-containing dog brain was passed intracerebrally to Swiss mice. When the animals became prostrate, the infected mouse brains were removed and stored in glycerin. Prior to each experiment, the glycerinated mouse brain virus is passed once intracerebrally through mice and the brains from the resulting prostrate animals are used to inoculate the test dogs. When the non-vaccinated dogs become prostrate, their brains in turn are passed intracerebrally through mice. The brains of these mice are removed when the animals become prostrate and stored in glycerin to serve as virus for the next dog experiment. Thus far, the infected mouse brains have continued to show large and numerous Negri bodies (Fig. 1).

Intramuscular Virulence for Dogs of Rabies Virus 15811

Experiments with various routes of injecting test virus into dogs resulted in the selection of the neck muscles as most natural and at the same time yielding the most measurable titration results. English investigators preferred this route as a result of 20 years' study of rabies vaccines (5). In our experience, titrations by the intracerebral route proved simple and gave quantitative results, but they were so severe that vaccinated dogs rarely withstood more than one lethal dose (6). Titrations by the lingual route likewise gave results capable of reproduction in our hands, provided the vaccinated dogs were lightly anesthetized and injected with virus into the same portion of the tongue and at the same depth. Titrations by the masseter muscle route gave less regular results in duplicate and repeated tests. Finally, titrations by the gastrocnemius muscle route provided data too variable for quantitative studies.

The results of titrations of rabies virus, No. 15811, into the neck muscles of beagle dogs, as described above, are shown in Tables I and II.

In Table I, the results of each test are set forth under the headings of dilution of virus injected, in terms of survival time in days of each fatal case, plus the number of survivors. Taken together, they reveal that in four tests in which the 1:50 dilution was given, a total of seventeen of

TABLE I

Neck Muscle Titrations of Dog Passage Virus, 15811, in 5 Months Old Beagle Dogs

Test	Fate of dogs injected with 0.25 cc. of virus into neck muscles of right and left sides in dilutions								
	1:50	1:100	1:200	1:400	1:500	1:2,500	1:4,000	1:10,000	1:40,000
P	15*, 16, 26								
1	14, 17, 17, 18, 22, 23				12, 14, 18				
2	11, 11, 11, 13								
3		12, 13, 14, 16			14, 18, S, S	17, 17, 17, 18			
4	13, 15, 15, 17				12, 14, 15, 27, 52			15, 18, 18, 23, 31	
5		12, 15, 17, 18, 18							
			12, 13, 14, 23, 34						
7			14, 15, 18, 18, 21, 22				16, 23, 39, S, S		22, 42, S, S
8				15, 18, S, S			16, 16, 16, 19, 19		22, S, S, S
10				12, 19, S			15, 16, 18, 30, S		39, 42, S, S
11			14, 16, S, S, S				18, 20, 22, 22, 33, S, S, S, S, S		S, S, S, S, S
Totals	17 of 17 = 100%	9 of 9 = 100%	13 of 16 = 81%	4 of 7 = 57%	10 of 12 = 83%	4 of 4 = 100%	17 of 25 = 68%	5 of 5 = 100%	5 of 17 = 29%
Combined totals	1:50 to 1:200 inclusive, 39 of 42 = 93%			1:400 to 1:10,000 inclusive, 40 of 53 = 75%					5 of 17 = 29%

* Day of death from rabies following injection.

S = remained well.

seventeen dogs succumbed (100 per cent); in two tests in which the 1:100 dilution was given, nine of nine (100 per cent), and in three tests in which the 1:200 dilution was given, a total of thirteen of sixteen dogs (81 per cent) succumbed. Again, in two tests in which the 1:400 dilution was given, four of seven (57 per cent) succumbed; in three in which the 1:500 dilution was given, ten of twelve (83 per cent); in one test with the 1:2,500 dilution, four of four (100 per cent); in four tests with the 1:4,000 dilution, seventeen of twenty-five (68 per cent), and in one test with the 1:10,000 dilution, five of five succumbed (100 per cent). Finally, in four tests in which the 1:40,000 dilution was given, five of seventeen (29 per cent) succumbed. Combining these figures again into groups in which the mortality was consistently: (*a*) close to 100 per cent; (*b*) less than 100 per cent but greater than 50 per cent, and (*c*) less than 50 per cent, we note that (*a*) dilutions of 1:50 to 1:200 inclusive resulted in a mortality of thirty-nine of forty-two tested dogs (93 per cent); (*b*) that dilutions of 1:400 to 1:10,000 produced a mortality of forty of fifty-three dogs (75 per cent), and (*c*) that dilutions of 1:40,000 brought about a mortality of five of seventeen tested dogs (29 per cent). From these figures we have taken dilution 1:200 as the least dose fatal to practically 100 per cent of test animals and dilution 1:10,000 as the least dose fatal to more than 50 per cent but less than 100 per cent.

TABLE II

Duration of Life of Dogs Following Injection of Dog Passage Rabies Virus, 15811, into the Neck Muscles of Young Beagle Dogs

Duration of life of injected dogs	Number and per cent of dogs succumbing at stated intervals following injection of virus in dilutions					
	1:50 to 1:200		1:400 to 1:10,000		1:40,000	
	No.	*per cent*	*No.*	*per cent*	*No.*	*per cent*
11 to 19 days	32/42	76	29/53	55	0/17	0
20 to 29 "	7/42	17	6/53	11	2/17	12
30 to 60 "	0	0	5/53	9	3/17	18
Survived 2 mos.	3/42	7	13/53	25	12/17	70

These end points are in keeping with figures obtained by grouping the durations of life of the dogs shown in Table I.

Table II shows that of forty-two dogs given the 1:50 to 1:200 dilutions of virus, 76 per cent died in 11 to 19 days, whereas of fifty-three given the 1:400 to 1:10,000 dilutions, 55 per cent succumbed and of seventeen given the 1:40,000 dilution, none succumbed within this 11 to 19 day period.

The percentages succumbing within 20 to 60 days were small (0 to 18) in all groups. Finally, of seventeen dogs given the 1:40,000 dilution, none died within the 11 to 19 day period, 30 per cent in the 20 to 60 day period, and 70 per cent remained well. In short, there is an increase in survival time as well as survival percentages at the 1:200, 1:10,000, and 1:40,000 dilution levels of virus.

Immunizing Potency of Commercial Canine Antirabies Vaccines

The above approximations of minimum lethal doses of virus were useful in evaluating our tests on dogs with commercial canine vaccines. At the outset, animals following vaccination were tested against two to four doses of virus fatal to nearly 100 per cent of controls—1:200 to 1:50 dilutions. Subsequently, however, in view of the failure of vaccinated animals to withstand this dose, they were tested against a much smaller amount of virus, namely, two to twenty minimum doses, fatal to more than 50 per cent but less than 100 per cent of controls—the 1:4,000 to 1:400 dilutions. These results likewise proved negative, as illustrated by the following series of protocols.

Experiment 1 (*Test 5*).—5 cc. of phenolized vaccine No. 4 were injected subcutaneously into each of five dogs; 5 cc. of No. 5 phenolized vaccine into five dogs; 5 cc. of No. 8 phenolized and 5 cc. of No. 9 each subcutaneously into each of five dogs respectively. Four dogs received 5 cc. of No. 1 chloroformized vaccine subcutaneously, and five dogs remained unvaccinated as controls. 3 weeks later each received 0.25 cc. of the 1:100 dilution of No. 15811 virus into the neck muscles of each side.

All five controls died of rabies on the 12th to 18th days following injection of test virus (100 per cent) (Table III). The five dogs receiving the No. 4 vaccine likewise died on the 9th to 26th days (100 per cent), and the five dogs receiving the No. 5 vaccine on the 12th to 18th days (100 per cent). Four of the five receiving the No. 8 vaccine died on the 12th to 19th days (80 per cent), and one survived; similarly, four of five receiving the No. 9 vaccine died on the 12th to 21st days (80 per cent), and one survived. In contrast to this, only one of the four dogs receiving the chloroformized vaccine No. 1 succumbed (25 per cent).

Table III shows that none of the phenolized vaccines protected dogs to the slightest degree against the 1:100 dilution of test virus, whereas the chloroformized vaccine afforded significant protection. The 1:100 dilution is regarded as two minimum doses fatal to 100 per cent of controls.

Experiment 2 (*Test 7*).—Six dogs, lot A, remained unvaccinated. Three dogs, lot B, each received 5 cc. of phenolized vaccine No. 3 subcutaneously. Six dogs, lot C, each received 5 cc. of chloroformized vaccine No. 3 subcutaneously. Five dogs, lot D, remained unvaccinated. Four dogs, lot E, received 5 cc. of phenolized vaccine No. 3 subcutaneously, and four dogs, lot F, remained unvaccinated. 3 weeks later, lots A, B, and C

each received 0.25 cc. of test virus diluted 1:200 into the neck muscles of each side. Lots D and E received the same dose of the 1:4,000 dilution, and lot F, the 1:40,000 dilution.

All six controls in lot A, receiving the 1:200 dilution, died of rabies on the 14th to 22nd days following injection (100 per cent) (Table IV). Similarly, two of the three in lot B receiving the phenolized No. 3 died on the 12th and 16th days (66 per cent), and five of six in lot C receiving the chloroformized vaccine died on the 13th to 23rd days (84 per cent). Three of five controls in lot D receiving the 1:4,000 dilution died on the 16th, 23rd, and 39th days respectively (60 per cent), and two of four in lot E receiving the phenolized No. 3 vaccine died on the 15th and 18th days (50 per cent). Finally, of the four control dogs in lot F given the 1:40,000 dilution, two died on the 22nd and 42nd days (50 per cent).

TABLE III

Immunizing Effects of Canine Antirabies Vaccines on Beagle Dogs

Experiment 1, Test 5

Treatment of dogs	Fate of dogs inoculated into the neck muscles (right and left) with 0.25 cc. of dog passage virus, 15811, diluted 1:100		
	Day of death following inoculation	No. dead/ No. injected	Dead
			per cent
A. No vaccine	12, 15, 17, 18, 18	5/5	100
B. Vaccine 4: Phenol, 5 cc. subc.	9, 10, 15, 18, 26	5/5	100
C. " 5: " " " "	12, 15, 16, 17, 18	5/5	100
D. " 8: " " " "	12, 13, 14, 19, S	4/5	80
E. " 9: " " " "	12, 13, 14, 21, S	4/5	80
F. " 1: Chloroform, 5 cc. subc.	13, S, S, S	1/4	25

S = animal remained well following injection. Survivors discarded after 60 days.

In this, as in the above experiment, there was no significant difference in the mortality of unvaccinated dogs given the 1:200 test dose (100 per cent) and in vaccinated dogs similarly tested (66 per cent and 84 per cent). Moreover, when the test dose was reduced to 1:4,000, a point close to the minimum dose fatal to 50 to 100 per cent, there was still no significant protecting effect of the vaccine,—controls 60 per cent as compared to vaccinated dogs 50 per cent.

Experiment 3 (*Test 8*).—Four dogs, lot A, remained unvaccinated. Each of three dogs, lot B, received 5 cc. of phenolized vaccine No. 9 subcutaneously, and four dogs, lot C, each received 5 cc. of chloroformized vaccine No. 1 subcutaneously. Five dogs, lot D, remained unvaccinated; three dogs, lot E, each received 5 cc. of phenolized vaccine No. 9 subcutaneously; four dogs, lot F, each received 5 cc. of phenolized vaccine No. 4 subcutaneously; and five dogs, lot G, each received 5 cc. of chloroformized vaccine

No. 1 subcutaneously. The final lot, H, remained unvaccinated. 3 weeks later each dog in lots A, B, and C received 0.25 cc. of test virus diluted 1:400 into the neck muscles of each side, each dog in lots D, E, F, and G, the same amount of virus diluted 1:4,000, and each dog in lot H, the same dose diluted 1:40,000.

Two of the four unvaccinated dogs of lot A died of rabies on the 15th and 18th days respectively (50 per cent). Two of the three vaccinated dogs in lot B died of rabies on the 13th and 14th days (66 per cent), and four of the four vaccinated dogs in lot C on the 15th, 17th, 19th, and 22nd days (100 per cent). Five of five unvaccinated dogs in lot D given the smaller dose of virus died on the 16th, 16th, 16th, 19th, and 19th days respectively (100 per cent). One of three vaccinated dogs in lot E died of rabies on the 19th day (33 per cent), three of four vaccinated dogs in lot F likewise succumbed on the 21st day (75 per cent), and two of five vaccinated dogs in lot G died on the 15th and

TABLE IV

Immunizing Effects of Canine Antirabies Vaccines on Beagle Dogs

Experiment 2, Test 7

Treatment of dogs	Dilution of test virus	Fate of dogs inoculated into the neck muscles (right and left) with 0.25 cc. of dog passage virus, 15811		
		Day of death following inoculation	No. dead/No. injected	Dead
				per cent
A. No vaccine	1:200	14, 15, 18, 18, 21, 22	6/6	100
B. Vaccine 3: Phenol, 5 cc. subc.	"	12, 16, S	2/3	66
C. " 3: Chloroform, 5 cc. subc.	"	13, 16, 17, 21, 23, S	5/6	84
D. No vaccine	1:4,000	16, 23, 39, S, S	3/5	60
E. Vaccine 3: Phenol, 5 cc. subc.	"	15, 18, S, S	2/4	50
F. No vaccine	1:40,000	22, 42, S, S	2/4	50

S = animal remained well following injection. Survivors discarded after 47 days.

22nd days (40 per cent). Finally, one of four unvaccinated dogs in lot H succumbed to the experimental injection on the 22nd day (25 per cent).

This experiment shows no protective effect of any vaccine. Lots A, B, and C received a dilution of 1:400, calculated as twenty minimum doses fatal to 50 per cent but less than 100 per cent. In this group, 50 per cent of the controls, A, died from the test injection, as compared with 66 and 100 per cent of the vaccinated B and C lots respectively. Again, lots D, E, F, and G received a still smaller test dose, 1:4,000, which is not more than two minimum doses fatal to 51 to 99 per cent. 100 per cent of the controls died, as compared to 33, 75, and 40 per cent of the vaccinated dogs in lots E, F, and G respectively. This 50 per cent or more mortality on the part of the vaccinated does not differ significantly from the average

figure for controls given this dilution (72 per cent), or indeed from the somewhat irregular figure (100 per cent) in this test.

Experiment 4 (*Test 10*).—Three dogs, lot A, five in lot B, and four in lot H remained unvaccinated. Each of five dogs in lot C received 5 cc. of phenolized vaccine No. 10 subcutaneously, and each of five in lot D, 5 cc. of chloroformized vaccine No. 1 subcutaneously. 3 weeks later, each dog of lot A received 0.25 cc. of virus 15811 diluted 1:400 into the neck muscles of each side, each of lots B, C, and D the same volume of virus diluted 1:4,000, and each of lot H, 1:40,000.

Two of the three control dogs, lot A, given the 1:400 dilution of virus died on the 12th and 19th days (66 per cent). Four of five controls, lot B, given the 1:4,000 dilution

TABLE V

Immunizing Effects of Canine Antirabies Vaccines on Beagle Dogs

Experiment 3, Test 8

Treatment of dogs	Dilution of test virus	Fate of dogs inoculated into the neck muscles (right and left) with 0.25 cc. of dog passage virus, 15811		
		Day of death following inoculation	No. dead/ No. injected	Dead
				per cent
A. No vaccine	1:400	15, 18, S, S	2/4	50
B. Vaccine 9: Phenol, 5 cc. subc.	"	13, 14, S	2/3	66
C. " 1: Chloroform, 5 cc. subc.	"	15, 17, 19, 22	4/4	100
D. No vaccine	1:4,000	16, 16, 16, 19, 19	5/5	100
E. Vaccine 9: Phenol, 5 cc. subc.	"	19, S, S	1/3	33
F. " 4: " " " "	"	21, 21, 21, S	3/4	75
G. " 1: Chloroform, 5 cc. subc.	"	15, 22, S, S, S	2/5	40
H. No vaccine	1:40,000	22, S, S, S	1/4	25

S = animal remained well following injection. Survivors discarded after 41 days.

of virus died on the 15th, 16th, 18th, and 30th days (80 per cent); four of five vaccinated dogs, lot C, given the same dilution of test virus, died on the 14th, 16th, 17th, and 43rd days (80 per cent), and two of the five vaccinated dogs, lot D, also given the same dilution of test virus, died on the 15th and 19th days (40 per cent). Two of the control lot, H, succumbed to the 1:40,000 dilution of virus (50 per cent).

In this test (Table VI), the mortality of the controls was 65 per cent as compared with 80 per cent and 40 per cent of the two vaccinated lots respectively, showing clearly, as in Experiments 2 and 3 above, that dogs vaccinated with commercial rabies vaccines do not withstand the least amount of test virus fatal to less than 100 per cent but more than 50 per cent of controls.

The results of all experiments to date on the immunizing potency of

commercial canine vaccines given to dogs according to directions are combined in Table VII. Of the groups receiving two to four times the least dose fatal to nearly 100 per cent of controls, the thirty-seven non-vaccinated animals showed a mortality of 100 per cent, the thirty-nine given phenolized vaccines, 82 per cent, and the thirty-six given chloroformized

TABLE VI

Immunizing Effects of Canine Antirabies Vaccines on Beagle Dogs

Experiment 4, Test 10

Treatment of dogs	Dilution of test virus	Fate of dogs inoculated into the neck muscles (right and left) with 0.25 cc. of dog passage virus, 15811		
		Day of death following inoculation	No. dead/No. injected	Dead
				per cent
A. No vaccine	1:400	12, 19, S	2/3	66
B. " "	1:4,000	15, 16, 18, 30, S	4/5	80
C. No. 10 phenolized vaccine	1:4,000	14, 16, 17, 43, S	4/5	80
D. No. 1 chloroformized vaccine	1:4,000	15, 19, S, S, S	2/5	40
H. No vaccine	1:40,000	39, 42, S, S	2/4	50

S = animal remained well following injection. Survivors discarded after 50 days.

TABLE VII

Summary of Mortalities of Dogs Vaccinated with Commercial Canine Vaccines and Tested Subsequently with an Intramuscular Injection of Dog Passage Virus, 15811

Vaccine employed	Mortality from test virus in dilutions			
	1:50 to 1:200		1:400 to 1:4,000	
	(2 to 4 × least dose fatal to 93 per cent of controls)		(2 to 20 × least dose fatal to 51 to 99 per cent of controls)	
	No.	*per cent*	*No.*	*per cent*
None	37 of 37	100	30 of 38	79
Phenol	32 of 39	82	9 of 13	70
Chloroform	19 of 36	53	4 of 10	40

vaccines, 53 per cent. Again, of the groups receiving a still smaller amount of test virus (two to twenty times the least dose fatal to less than 100 per cent but more than 50 per cent of controls), the thirty-eight non-vaccinated animals showed a mortality of 79 per cent, the thirteen given phenolized vaccine, 70 per cent, and the ten given chloroformized vaccines, 40 per cent. These differences between vaccinated and non-vaccinated dogs are negligible.

Immunizing Potency of Commercial Canine Antirabies Vaccines Administered in Larger Doses and by the Intraperitoneal Route

Concurrently with the above experiments, tests have been made of the immunizing potency of canine vaccines administered in larger doses and by the intraperitoneal rather than the subcutaneous route. Previous tests in mice (1) had shown that chloroformized vaccines are capable of conferring a degree of immunity, provided two to five times the comparable dose per gram of body weight is given and the injections are made intra-

TABLE VIII

Immunizing Effects of Canine Antirabies Vaccines on Beagle Dogs

Experiment 5, Test 4

Treatment of dogs	Dilution of test virus	Fate of dogs inoculated into the neck muscles (right and left) with 0.25 cc. of dog passage virus, 15811		
		Day of death following inoculation	No. dead/No. injected	Dead
				per cent
A. No vaccine	1:50	13, 15, 15, 17	4/4	100
B. Vaccine 3: Chloroform, 1 dose, 20 cc., iper.	"	34, 36, S, S, S	2/5	40
C. " 3: Chloroform, 1 dose, 10 cc., iper.	"	S, S, S, S	0/4	0
D. " 3: Chloroform, 2 doses, 5 cc. each, iper.	"	22, S, S, S	1/4	25
E. Vaccine 3: Chloroform, 1 dose, 10 cc., subc.	"	22, 23, 54, S, S	3/5	60
F. " 3: Chloroform, 2 doses, 5 cc. each, subc.	"	19, S, S	1/3	33
G. No vaccine	1:500	12, 14, 15, 27, 52	5/5	100
H. " "	1:10,000	15, 18, 18, 23, 31	5/5	100

S = animal remained well following injection. Survivors discarded after 70 days.

peritoneally instead of subcutaneously. The following protocol illustrates the type of experiment and results obtained in dogs.

Experiment 5.—Four dogs, lot A, remained unvaccinated. Five dogs, lot B, received chloroformized vaccine No. 3 in one dose of 20 cc. intraperitoneally. Four dogs, lot C, received the same vaccine in one dose, 10 cc., intraperitoneally; four dogs in lot D received the vaccine in two doses of 5 cc. each intraperitoneally; five dogs in lot E each received one dose, 10 cc., subcutaneously, and three dogs, lot F, two doses of 5 cc. each subcutaneously. Five dogs in lot G and five in lot H remained unvaccinated. 3 weeks later each dog in lots A to F inclusive received 0.25 cc. of virus 15811, diluted 1:50, into the neck muscles of each side. Dogs in lot G received the same dose of a 1:500 dilution, and lot H, a 1:10,000 dilution.

The results of the test are shown in Table VIII. The lot A controls died of rabies on

the 13th, 15th, 15th, and 17th days respectively (100 per cent). Two of the five dogs in lot B, given 20 cc. of vaccine intraperitoneally, died on the 34th and 36th days (40 per cent). No dogs in lot C, given 10 cc. of vaccine intraperitoneally, succumbed (0 per cent). Only one of four dogs in lot D given two 5 cc. doses intraperitoneally died (25 per cent). Three of five dogs in lot E, given 10 cc. of vaccine subcutaneously, died on the 22nd, 23rd, and 54th days respectively (60 per cent). One of three dogs given two doses of 5 cc. each subcutaneously died on the 19th day (33 per cent). Five controls given the 1:500 dilution of test virus died on the 12th, 14th, 15th, 27th, and 52nd days (100 per cent). Five dogs given the 1:10,000 dilution of test virus likewise died on the 15th, 18th, 18th, 23rd, and 31st days (100 per cent).

The test shows (Table VIII) that chloroformized vaccine immunizes dogs fairly well (ten of thirteen, 77 per cent), if given intraperitoneally in at least double the standard 5 cc. dose. If given subcutaneously in similarly large doses, the result, although less striking (four of eight, 50 per cent), is still significant. This protection was obtained against a large amount of test virus—in this single test, at least 200 times, although in average tests not more than four times, the least dose fatal to 100 per cent.

DISCUSSION

The development of a quantitative method for measuring the immunizing potency of antirabies vaccines in dogs affords an opportunity to test rabies vaccines critically under conditions approaching those in nature. The failure of commercial phenolized and chloroformized canine vaccines to immunize dogs under the above experimental conditions weighs against their present value in the field. On the other hand, the fact that chloroformized vaccines in larger doses, administered intraperitoneally, successfully immunize suggests that the development of a potent, practical vaccine is not an impossibility.

The dog test has likewise been useful in checking the results of the mouse test (1). Phenolized vaccines found to be negative in mice proved likewise negative in dogs and chloroformized vaccines, equivocal or irregular in mice, proved the same in dogs. Finally, larger doses of chloroformized vaccines injected intraperitoneally proved effective, though irritative, in both mice and dogs. Thus the results of the dog test have paralleled those of the mouse test.

The parallelism between results in dogs and those previously reported in mice (1) establishes the reliability of the mouse test as an index of the immunizing effect of rabies vaccines in other animal species. Accordingly the mouse test becomes a simple, inexpensive, and practical tool for the testing of routine or experimental vaccines.

CONCLUSIONS

1. A quantitative method is described for testing the immunizing potency of antirabies vaccines in dogs.

2. Phenolized, single-injection, canine vaccines from seven manufacturers, when administered to dogs according to directions, failed to protect them against the least measurable amount of test virus fatal to 50 per cent or more of controls. Chloroformized vaccines from two of three manufacturers, under the same conditions, gave equivocal or suggestive results.

3. Commercial chloroformized vaccines in 10 cc. doses, injected intraperitoneally rather than subcutaneously into dogs, conferred a significant degree of immunity but proved temporarily irritative to the peritoneum.

4. These results of canine vaccines in dogs parallel closely those already reported in mice.

BIBLIOGRAPHY

1. Webster, L. T., *J. Exp. Med.*, 1939, **70**, 87.
2. Webster, L. T., *J. Am. Vet. Med. Assn.*, 1940, **96**, 65.
3. Webster, L. T., Third International Congress for Microbiology, Abstracts of Communications, New York, 1939, Section III, 108.
4. Casals-Ariet, J., and Webster, L. T., *J. Bact.*, 1940, **39**, 66.
5. Webster, L. T., *Am. J. Hyg.*, 1939, **30**, Section B, 113.
6. Webster, L. T., *Am. J. Pub. Health*, 1938, **28**, 44.

EXPLANATION OF PLATE 35

FIG. 1. Negri bodies in Ammon's horn of W-Swiss mouse injected intracerebrally with the brain of a non-vaccinated dog in Experiment 10, which had been inoculated intramuscularly with rabies strain 15811.

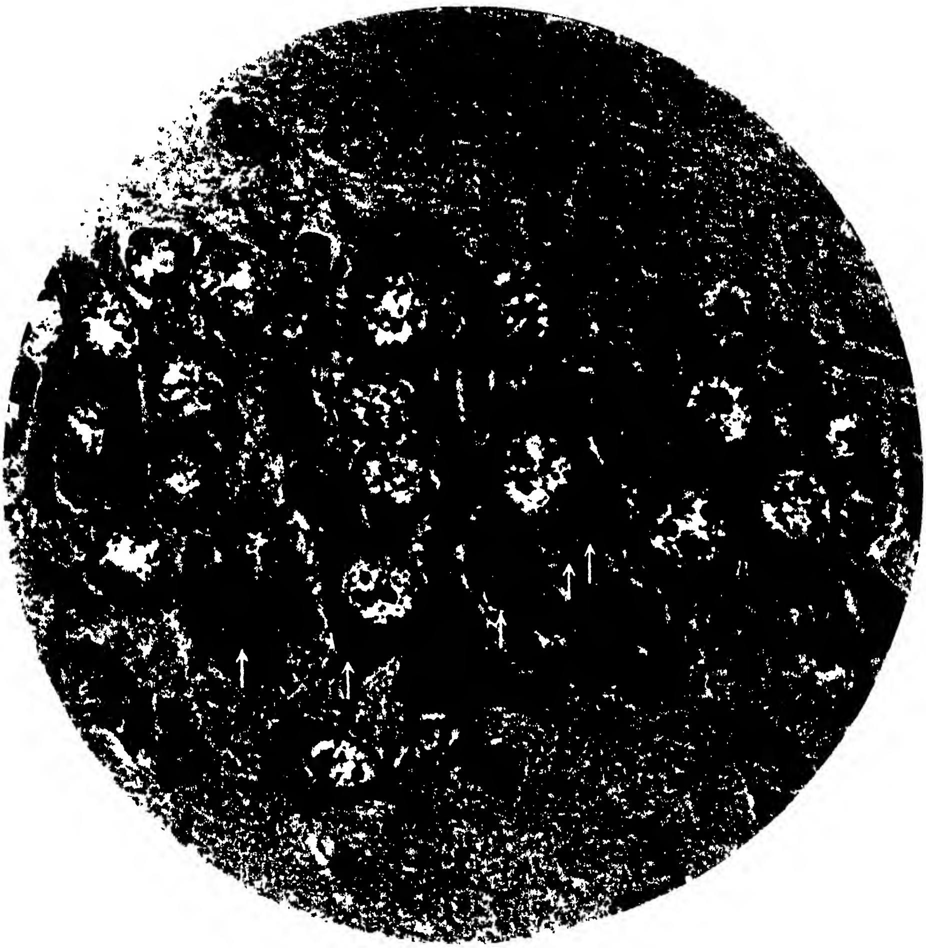

FIG. 1

Photographed by Joseph B. Haulenbeek

(Webster and Casals: Immunizing potency of antirabies vaccines)

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THE ACTIVATING, TRANSFORMING, AND CARCINOGENIC EFFECTS OF THE RABBIT PAPILLOMA VIRUS (SHOPE) UPON IMPLANTED TAR TUMORS

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Plates 36 to 41

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The virus causing cutaneous papillomas in cottontail rabbits (1) produces remarkable effects when injected into the blood stream of domestic rabbits carrying benign tar tumors (2). It localizes in many of these growths, converts not a few into virus papillomas, makes others proliferate with far greater rapidity than before, alters some with result in tumors of unusual sort, and causes a considerable proportion to take on the character of progressive cancers when otherwise they would remain benign and ultimately disappear. The work now to be recorded has been carried out in the further study of these phenomena. To determine directly the effects of the virus on individual tar tumors, pieces of many of them of various sorts were exposed to it *in vitro* and implanted in the muscles and subcutaneous tissue of the hosts.

Numerous viruses are known to be capable of flourishing within tumors, but none except the one causing rabbit papillomas has been observed to have any important influence upon the growths. Years ago Levaditi and Nicolau demonstrated that extraneous viruses may exist in permanent association with transplanted neoplasms into which they have been experimentally introduced (3). Levaditi and his associates have since shown that herpes, vaccinia (4), rabies (5), and fowl pest virus (6), and the virus of lymphogranulomatosis (7) all have this ability. Virus lymphogranulomatosis gives rise to the corpuscles typical of its action, and herpes, rabies, and vaccinia virus produce their characteristic inclusion bodies. In an early paper Levaditi and Schoen stated that vaccinia infection may at first cause tumors to grow faster, but there is no mention of this phenomenon in later communications, and other workers seem not to have observed it. The virus of fowl pest destroys the cells of transplantable mouse tumors, but has no effect on those of the Brown-Pearce rabbit tumor, though persisting in the growth. Findlay and MacCallum (8) have demonstrated that several strains of yellow fever virus may be carried along in mouse carcinomas, their sole action being to increase necrosis in certain instances. Pownall and Florey (9) reported that the dermotropic variety of the virus of infectious ectromelia retards or destroys tar tumors in mice; but it is uncertain whether this is consequent on virus infection of the cells of the growths or due to the serious damage done to the skin round about them. Rivers and Pearce

(10) have contributed the significant observation that transplanted tumors may accidentally become infected with an extraneous virus. They encountered virus III as a contaminant of Brown-Pearce tumors. It gave no morphological sign of its presence other than a few inclusion bodies. Mellanby (11) found that the tar and benzpyrene tumors of fowls may become infected with the virus causing a chicken sarcoma (chicken tumor I), when a growth of this sort is produced in them, the infection taking place during the period when the virus of the enlarging sarcoma circulates in the blood. It brings about no discernible change in the tar and benzpyrene tumors, is present in only small amounts, and can no longer be procured from the growths after they have been transplanted once or twice. Andrewes and Ahlström (12), studying a rabbit sarcoma which arose secondarily in a connective tissue mass resulting from localization of the Shope fibroma virus in voluntary muscle injected with tar, made numerous tests to learn whether the virus accompanied the sarcoma on serial transplantation. It was not demonstrable either directly or on immunological test. Another sarcoma that appeared under the same circumstances failed to grow on transplantation (13).

Morphological evidence of the formative influence of the rabbit papilloma virus can be discerned in many of the cancers springing from the growths it produces (14); and the persistence and increase of the virus have been demonstrated in two such cancers which were successfully transplanted, one of them to ten successive groups of animals (15). The virus fails to establish itself, however, in Brown-Pearce rabbit carcinomas into which it has been experimentally introduced (16).[1] The benign papillomas it directly causes are themselves susceptible to infection with other viruses. Levaditi and Schoen proved that vaccinia virus will localize out of the blood stream and persist in the papillomas (17); and Syverton and Berry have demonstrated that the papilloma cells and those of the derivative cancers will support two viruses at once in addition to that which is the cause for their pathological state (18). The latter investigators utilized both cottontails and domestic rabbits, when studying the multiple virus infections, and inoculated the growths with herpes and vaccinia, B virus, virus III, and *Virus myxomatosum*, employing as telltales the characteristic cytoplasmic and intranuclear inclusion bodies which these agents produce, and utilizing serological tests as well.

Materials and Methods

For the purposes of the present work tumors elicited with tar were partly or wholly excised and cut fine. Two equal portions of the fragments were placed in Tyrode solution containing virus and ordinary Tyrode respectively at pH 7.2, and after a brief interval they were implanted at corresponding situations in the subcutaneous connective tissue and leg muscles of the animals from which they had come.

The Procedure with Domestic Rabbits.—The domestic rabbits providing tumors were all of brown-gray (agouti) breed, and had been tarred twice a week on the insides of

[1] The Brown-Pearce tumors developing from implanted tissue which had been exposed *in vitro* to the virus grew much less rapidly than did those from material merely exposed to Tyrode solution. In later unpublished experiments of the sort no such antithetic effect of the virus has been noted.

the ears during some weeks or months, with intermissions in the case of a few of those tarred longest.[2] In many instances tarring was kept up for from 1 to 3 weeks after the implantations because of the possibility that the general changes it brought about might favor growth at the new sites. No indication of such influence was discernible in the behavior of the implants.

Early experiments showed that if the tumors were utilized immediately after they had been stripped of tar, abscesses were likely to develop at the implantation sites because of bacteria in the moist, and often macerating, growths. If the tar was removed a few days before the experiment, however, the tumors dried down and could be used with little risk of purulence. Hence in the later work this was regularly done. The growths were punched from the ear with sterilized cork borers, a single blow of the mallet sufficing; and whenever they were large enough a piece was left *in situ*. This was a great desideratum as disclosing the capabilities of the tumors at situations where they were already established. Bacterial infection of the wounds almost never followed, and the holes in the ears healed in rapidly. A median slice of each disc punched out was put into Zenker's fluid for section, and the remaining tumor tissue was trimmed free from normal elements, so far as possible, and its basal portion was sliced off and cut fine in a dish containing a little Tyrode to which sometimes a few drops of the animal's own serum had been added with a view to cell protection. This precaution seemed to make no difference in the results, nor was any to have been expected since previous implantation experiments with virus papillomas and the derivative carcinomas had abundantly shown that Tyrode brought to pH 7.2 with carbonic acid gas is not injurious to tumor cells exposed to it for brief periods.

The hashed material was separated into two lots of equal size, which were taken up separately on the blade of a knife and transferred to two graduated, 15 cc. centrifuge tubes, one containing 1 cc. of Tyrode solution, the other 1 cc. of a virus-containing Berkefeld filtrate of a Tyrode extract of a cottontail papilloma. Sometimes serum had been added to the fluids for the reason just given. The tubes were kept at room temperature for a period ranging from 15 to 25 minutes, and whirled between the hands at intervals to resuspend the fragments. Then Tyrode was added to the 15 cc. mark, they were suspended once more, and sedimented at once by brief centrifugation. The supernatant fluid was pipetted away as completely as possible, and enough new Tyrode was put on so that 8 to 15 fragments, suspended in 1 cc. of fluid, could be injected at one or two places in the host. The spots utilized were the upper extensor muscles of all four legs and the subcutaneous tissue of the axillae and groins. Sometimes the materials were put at two situations in each groin. The skin was slit prior to introduction of the injecting needle, to exclude the possibility that normal epithelium might be carried in on its point, but no precaution was taken to avoid virus infection of the epidermal wound when the needle was withdrawn, and papillomas frequently appeared in the newly healed scar, showing that a superabundance of virus had been present in the injected suspension. There was never any evidence, though, that it reached distant points in the body. The injections were done forcibly to separate the fragments, an aim accomplished as the eventual sections showed; and afterwards some of the virus was rubbed into a freshly scarified patch of skin on the rabbit's side, to determine its pathogenicity. Confluent papillomatosis regularly resulted. The implantations took but a

[2] Horizontal retort tar was used, from the Ostergasfabrik of Amsterdam. It was the gift of Dr. Karl Landsteiner.

few minutes and the control specimens,—which had been held *in vitro* for the same time as the experimental,—were injected first.

Usually there were many benign tar tumors on the ears of the rabbits, and tissue from the four or five largest was submitted to the action of the virus, as was also a piece of the hyperkeratotic epidermis, punched from a spot where no growths were visible, trimmed of cartilage and connective tissue, and hashed in the usual way. Ordinarily the animals were killed within 3 to 6 weeks since our aim was to learn the early changes produced by the virus, as distinct from such alterations as might take place secondarily in the enlarging growths.

The Procedure with Cottontails.—The ears of the cottontails had generally been tarred longer, for several months at least, sometimes for a year or more, and often on both surfaces. Only cancerous growths were utilized. A piece was punched out of one such tumor of each animal, and cut up, treated in the way already described, and implanted at four to six corresponding situations in the leg muscles and subcutaneous tissue. The tumors had usually ulcerated, and abscesses often complicated the outcome.

Results with the Tar Tumors of Domestic Rabbits

The benign tumors evoked in domestic rabbits by tar are of three well defined types (19). We have utilized examples of all three,—the rare frill horns and the more rapidly growing tar papillomas and carcinoids. The tar carcinoids, so designated by Borst (20), might better be called carcinomatoids,[3] to avoid confusion with the argentaffin tumors to which the term carcinoid is currently applied. They are growths having the histology of carcinomas and often ulcerating and extending through the ear and into lymph vessels, yet they are actually examples of spurious malignancy induced by local conditions (19). Their cancerous traits are wholly dependent upon the stimulation provided by continued tarring, and after this has been left off they either disappear or round up into epidermal cysts or become ordinary, benign papillomas. Proof exists (19) that they are intrinsically tumors of the latter sort, with cells notably responsive to stimulating influences and for this reason mimicking cancer cells when tarred.

None of the control implants of 57 tar tumors gave rise to a growth at the new situation (Table I). The tumor tissue either disappeared promptly, or died by keratinization,—with persistence for a while of the keratinized fragments,—or gave rise to minute, keratinizing cysts lined with stratified squamous epithelium, like those arising from implants of ordinary skin. In this respect our findings agree with those of Ferrero (21) who noted the failure of benign tar tumors to grow after implantation in the host rabbit.

The virus regularly "took" on the bits of epidermis punched from skin rendered hyperkeratotic by tarring but devoid of tumors. There resulted

[3] Suggestion of Dr. H. T. Karsner.

nodules of papilloma tissue precisely like those which develop after the transfer to the muscles or connective tissue of fragments of virus papillomas produced by infecting normal skin (22). As in such instances, the growths consisted of more or less spherical cysts, often partially fused or multilocular, frequently patched with gray, and having a solid, lamellated core of dead, keratinized cells surrounded by a layer of living, differentiating

TABLE I

Effects of the Papilloma Virus on the Benign Tar Tumors of Domestic Rabbits

Number of rabbits implanted	Tarred	Interval to implantation	Later tarring	Period of implantation	The implanted tumors		Outcome of implantation	
					Character	Fate on ears	Tyrode-steeped materials	Virus-steeped materials
	days	*days*	*days*	*days*				
7	78	5 to 10	9 to 21	20 to 64	12 benign papillomas	9 excised 1 vanished 1 stationary 1 grew	10 negative 2 tiny cysts	1 negative 1 tiny cyst 10 virus papillomas
					20 carcinomatoids	5 excised 9 vanished 2 dwindled 4 became benign papillomas	19 negative 1 tiny cyst	9 negative 8 virus papillomas 1 carcinomatoid (?) 2 carcinomas
1	91	2	21	59	1 frill horn	Excised	Negative	Tiny cyst
					3 benign papillomas	1 excised 2 stationary	2 negative 1 tiny cyst	1 negative 2 virus papillomas
3	112	275	None	42 to 65	2 frill horns	Excised	Negative	2 negative
					11 papillomas*	10 excised 1 stationary	Negative	7 negative 4 virus papillomas
2	200	92	7	38	8 benign papillomas	6 excised 1 dwindled 1 grew	7 negative 1 tiny cyst	3 negative 3 virus papillomas 2 tar papillomas

* 8 of the papillomas had been slowly retrogressing, 1 was stationary, and 2 were slowly enlarging.

epithelium. Often living papillae existed within the core, and always the proliferating cell layer exhibited the cytological peculiarities characteristic of the virus' action (19). Growth was often extremely rapid, the nodules reaching a diameter of several centimeters within a few weeks.

Pieces of three *frill horns* were exposed to the virus, all of them small tumors which had enlarged slowly. The control fragments of these growths disappeared, whereas those that had been exposed to virus yielded in one instance a virus papilloma and in another a tiny cyst lined with the typical,

frill horn epithelium. Incidentally to previous work (2) we have repeatedly noted the conversion of frill horns into papillomas after the virus had reached them *in situ*. Their cells, which have a highly characteristic morphology, were directly converted into virus papilloma cells, exhibiting the stigmata distinctive of such elements (19).

Of 34 *tar papillomas*, 15 gave no evidence of any effects of the virus, the tissue exposed to it either vanishing, or keratinizing completely, or rounding up into tiny cysts lined with stratified squamous epithelium, like some of the control implants. The tissue of 17 other growths gave rise to nodules of what appeared in the gross to be ordinary virus papillomatosis. As already mentioned, tissue of this sort often proliferates with prodigious rapidity after implantation within the body, and one would expect it to outgrow tar papilloma tissue in mixed grafts unless this latter was greatly stimulated by the virus. Most of the nodules appeared to consist entirely of virus papilloma but microscopic search sometimes disclosed living, proliferating islands of the tar tumor. The two sorts of papillomatosis can be readily distinguished under ordinary conditions owing to their cytological peculiarities (19), but this was not so easy in implantation nodules of mixed composition because the epithelial layers of differing kinds had frequently united and appeared to grade into each other. The longer the interval before the animal was killed, the less often was tar papilloma tissue encountered, for the reason that virus papilloma tissue more and more preponderated.

Fragments of two tar papillomas which had been exposed to the virus established themselves on implantation and grew actively while retaining their distinctive character.

D. R. 3-73 provided both instances (Table I). They were essentially similar. One is illustrated in Figs. 1, 2, and 3. As Fig. 2 shows, each of the several bits of tumor implanted in a leg muscle after exposure to virus gave rise to a papillomatous growth with the traits of the original tar tumor (Fig. 1). The orientation of the epithelial layer was the reverse of that in nodules consisting of virus papilloma tissue. When growing within the body the latter forms cysts filled with keratinized material and enclosed in a rind of living epithelium which differentiates *inwards* (Fig. 3). The opposite held true of the tar papilloma tissue submitted to the influence of the virus; its epithelium formed knobs connected with the host by pedicles, and these knobs keratinized *outwards*, soon becoming surrounded by much dead, squamous material (Fig. 2). The tar papilloma of Fig. 1 had been completely excised and the fragments implanted as controls disappeared.

The results with the other tar papilloma stimulated by the virus differed only to the extent that the piece of tumor left on the chronically inflamed ear continued to grow. The effect of chronic inflammation to bring about a persistence of tar papillomas

which would otherwise vanish has been stressed in a previous paper (2). Each of the numerous fragments implanted after exposure to the virus rounded into a keratinizing knob and grew, whereas the control fragments failed to establish themselves.

Carcinomatoids are to be found only on ears which have recently been tarred (19), and hence none was available in the case of the rabbits utilized for our experiments long after the last tarring. Bits of 20 carcinomatoids in all were subjected to virus infection (Table I). During the days before they were taken, when no more tarring was done, the growths dried down and in some instances began to involute. The virus had no evident effect upon 9 of them, gave rise to nodules of virus papillomatosis in 8, and in 3 caused tumors to appear which were invasive and had the morphology of carcinomas. One of these last consisted in the main of tissue like that of the original carcinomatoid, but much virus papillomatosis was present as well. This complication was negligible in one of the other two tumors and absent from the third. The findings with them were as follows:—

The carcinomatoid of D. R. 4-97 was an ulcerated disc 1 cm. across when about half of it was taken for implantation (Figs. 4 and 5). The remainder dwindled and had vanished when the animal died 20 days later. During this period a nodule 1.5 cm. across, of cancerous tissue devoid of papillomatous characters (Fig. 6), formed in the axillary connective tissue where bits of the virus-infected material had been placed. The control fragments disappeared.

Figs. 7 to 9 summarize events in the case of a growth of D. R. 5-10. The carcinomatoid furnishing the material was 2.3 cm. in longest diameter, a raised, raw disc which had extended, near its middle, to the outside of the ear through lacunae in the cartilage. About one-tenth of the disc was removed for implantation 5 days after the tar had been stripped from the ears after 78 days of tarring. This was resumed for 11 days after the biopsy and then discontinued for good. During the 56 days before the animal was killed the ear tumor gradually vanished. A slice taken at autopsy through the mound which marked where it had once been showed ordinary, stratified squamous epithelium, smooth and slightly hyperplastic, covering redundant connective tissue, with a patch of foreign body giant cells deep in the latter round about a few dying tumor cells (Fig. 8). Nothing was found in the leg where the control fragments had been implanted, whereas the material exposed to virus had given rise to an actively invasive growth 2 cm. in diameter. Multicentric, because of proliferation from the scattered tumor fragments, it was more malignant-looking (Fig. 9) than the original carcinomatoid (Fig. 7). A small, discrete island of Shope papillomatosis was present in the growth at one spot.

The character of the highly invasive, anaplastic, progressively enlarging neoplasms which resulted from the action of the virus upon carcinomatoids leaves no doubt that these had been rendered cancerous. The growths wholly resembled some of those fatal and frequently metastasizing tumors

resulting from the action of the virus upon tar papillomas and carcinomatoids situated on the tarred ear (2). Virus papilloma tissue, after transfer to muscle, occasionally invades and simulates carcinomatosis in the brief period before its epithelium differentiates and forms multilocular cysts. But even at such times its cytology is very different from that of the growths just considered.

The Results with Old Tar Tumors

Facts already reported (19) have shown that the benign tar tumors elicited by tarring the ears of domestic rabbits are all conditional growths devoid of the power to proliferate without the aid provided by local conditions. Tarring induces these conditions, rendering them permanent if it is long kept up, and growths thus aided may persist and enlarge, and occasionally change into cancers. The longer tar is applied the more likely is this to happen. It has seemed possible, in view of such findings, that benign tar tumors might gain the ability after a while to persist without aid, and to grow upon transfer to other situations. To obtain light on this point we have carried out implantation experiments with 19 papillomas which had been present for months.

Three of the animals furnishing the growths had been tarred for 112 days and then kept for 275 days more, during which period certain of their larger papillomas had persisted on the much altered ears, a few growing slowly, a few remaining unchanged, while the majority slowly dwindled. The eleven growths which were most vigorous were utilized for test, and two frill horns as well. The results with the latter have already been mentioned. Neither they nor the papillomas manifested any ability to grow on implantation, and exposure of their hashed tissue to the virus led at most to virus papillomatosis.

Eight tar papillomas were utilized which had persisted for 92 days after a tarring period of 200 days. They came from two animals. Again the control implants failed to grow, but in two instances the fragments exposed to infection with the virus proliferated actively at the new situation and retained the characteristic morphology of tar papillomas. These instances have also been described.

Effects of the Virus on the Tar Cancers of Wild Rabbits

The tar we employed very seldom elicits cancers in domestic rabbits and then only after a year or more; but it frequently calls them forth within a few months in cottontails. Experiments were carried out with some of the tumors thus provided.

The cancers evoked by tar in cottontails are often multiple, especially when it has been applied to both surfaces of the ear, a procedure well sustained, though soon fatal in our experience to most domestic rabbits. The tumors utilized were squamous cell carcinomas, as proven by their morphology, by continued growth of the portions left

behind on the ears, sometimes by metastasis, and by growth of the control implants in certain cases. Ulceration had usually taken place, necessitating the use of the most deep-lying tissue. Occasionally the cancer occupied so much of the organ that amputation was done instead of a punch biopsy. Metastases in lymph nodes at the base of the ear were taken as material in two cases. Despite these precautions abscesses totally destroyed the grafts in three of twelve implanted animals, and complicated the findings in most of the others. Tarring was stopped after the implantations.

Only equivocal results were obtained with the metastatic tissues. In one case abscesses destroyed all of the grafts except one,—which had been exposed to the virus,—and where this had been put a small area of unhealthy carcinomatosis was present in addition to an abscess. In the other animal a single, minute nodule of unhealthy squamous cell carcinomatosis was found,—also at a site where tumor bits exposed to the virus had been placed. This rabbit was not killed until 72 days had elapsed.

One of the remaining seven animals died on the 10th day after implantation. Papillomas could not be seen as yet on the inoculated area on its side, and microscopic sections were not taken, so there is no certainty that the rabbit was susceptible to the virus, since some "normal" cottontails prove refractory on inoculation, presumably because of previous infection (23). The cancer had just begun to grow at all of the implantation sites and no influence of the virus upon it could be discerned. Nor was any evident in the growths of two animals which lived longer and developed papillomatosis where the skin had been inoculated. One of them, killed on the 25th day, had small nodules of moderately anaplastic squamous cell carcinoma, together with small abscesses, at every implantation site. In the other the cancer failed to survive at any of the five control sites, and barely did so at two situations where material exposed to virus had been put, while at the remaining three large nodules of ordinary virus papillomatosis were found at autopsy on the 62nd day. There had been opportunity in this instance for the virus to infect non-neoplastic epidermis included in the tumor fragments.

The virus had remarkable effects upon the cancers of four animals (Table II).

W. R. 100 E.—This animal was subjected to two courses of tarring, on both sides of the ears, throughout two periods totaling 7½ months in all, with an interval of 3 months. The cancer appeared at the end of the second period, grew rapidly on both sides of the ear, and after another 2 months had occupied its whole further half. The tumor was 1 cm. thick on the inner side of the cartilage, fleshy and ulcerated, with a rolled rim, and on the outer side was nearly as thick, nodular, partly ulcerated, but with a subcutaneous extension. A part of this latter was punched out and utilized. The growth grated under the knife, showed numerous yellow, opaque dots, and proved to be a cystic, squamous cell carcinoma (Fig. 10), orderly for the most part and keratinizing, but in some places breaking up into small nests of anaplastic cells. Five implantations were made of the control fragments and of fragments exposed to virus, respectively. When the animal was killed, after 2 weeks, large cancerous nodules had arisen (Fig. 12) wherever the latter had been put (Table II), while nothing was found at one of the control situations, at three others the tumor had barely succeeded in surviving, and at the fifth site a minute cancerous nodule had formed (Fig. 11). There were abscesses amidst some of the large tumors due to the virus-infected material. The cancer on the ear had continued to grow, but it had not metastasized.

In this instance the virus not only stimulated the cancerous tissue, enabling it to proliferate with great rapidity (Table II), but exerted a

TABLE II

Effects of the Papilloma Virus on the Tar Cancers of Cottontail Rabbits

Rabbit No.	Character of implanted material and fate of cancer on ear	Implantation period	Situation of implant	Tumors resulting from tissue exposed to: Tyrode — Size	Tyrode — Constituents	Virus — Size	Virus — Constituents	Virus yield of: Tyrode growths	Virus yield of: Virus growths	Remarks
		days		*cm.*		*cm.*				
100 E	Nodular, fleshy growth covering half of an ear: a differentiating, cystic, squamous cell carcinoma (Fig. 10)	14	Foreleg	0.5	Small abscess and minute cysts lined by unhealthy tumor cells	1.8	Original cancer with increased anaplasia, and *new hybrid cancer*			See Figs. 1[illegible] 14
			Axilla	0.2	Keratinized cysts and one minute cancer nodule	0.5	*New hybrid cancer*			
	(Growth continued to enlarge)		Groin	0		1.8	*New hybrid cancer*			
			Anterior thigh	0.8	Abscess with a few cancer islands in wall	2.8	Much *new hybrid cancer* in wall of large abscess			
			Posterior thigh	0.5	Abscess with a few cancer islands in wall	1.5	Large nodules of *new hybrid cancer* and small abscess			
1-47 E	Small, fleshy, ulcerated squamous cell carcinoma, moderately anaplastic	19	Foreleg	2.7	Large abscess with some cancer of original sort	2.7	Scattered abscesses in large mass of original cancer	0	±	
			Axilla	1.3	Abscess only	1.3	Abscess only			
	(Growth recurred at edge of punch hole)		Anterior groin	1.5	Abscess only	1.3	Original cancer; *new hybrid cancer*		±	
				1.7	Original cancer	1.7	Original cancer; *new hybrid cancer*			
			Posterior groin	0.6	Cyst lined with unhealthy cancer cells	0				
			Anterior thigh	2.0	Original cancer	2.3	Original cancer	0	0	
			Posterior thigh	1.5	Original cancer	1.4	Original cancer	0		
	Inoculation papilloma on side							+		

TABLE II—*Concluded*

Rabbit No.	Character of implanted material and fate of cancer on ear	Implantation period	Situation of implant	Tumors resulting from tissue exposed to: Tyrode — Size	Tyrode — Constituents	Virus — Size	Virus — Constituents	Virus yield of: Tyrode growths	Virus yield of: Virus growths	Remarks
		days		*cm.*		*cm.*				
1-40 E	Large, fleshy, ulcerated squamous cell carcinoma, keratinizing and cystic (Ear amputated)	26	Foreleg	0.15 0.15 0.2	Original cystic cancer, beginning to invade	2.5	Separate regions of virus papilloma and original cancer; of an intimate mixture of the two; and of a *new hybrid cancer.* Also abscess 0.6 cm. across		++	Serum at death contained antibody in considerable amount
			Axilla	1.3	Abscess only	1.5	Virus papilloma in wall of abscess		++	
			Anterior groin	0		1.7	Virus papilloma. Original cancer lying separate		++ +	
			Posterior groin	0		1.4	Virus papilloma and original cancer closely intermixed		++± +++	Twice tested
			Anterior thigh	3.5	Large abscess with a little cancer in wall	1.3	Virus papilloma and original cancer closely intermixed			
			Posterior thigh	0.4	Original cancer, invading	1.4	Original cancer *New hybrid cancer*	0	±	
	Inoculation papilloma on side							++++		
61 N	Subepidermal tumor consisting of three different types of squamous cell carcinomatosis (Figs. 18-20) (Growth continued to enlarge)	72	Foreleg	0		4 × 3	Virus papilloma		+	
			Axilla	0		1.3 2.0	Some cancer of original sorts but mostly virus papilloma Mostly cancer of all three original types; a little virus papilloma tissue		±	
			Groin	0.1	Cyst lined with dead epithelium	1.3	Mostly original cancers but some virus papilloma			See Figs. 15-20
			Thigh	0.2	Original cancer, unhealthy	5 × 3.5	Original cancers. Virus papilloma in about equal amount		0 0	
	Original cancer as above							0		
	Inoculation papilloma on side							+±		

formative influence upon it (Figs. 12 and 14), rendering it unlike both the original tumor (Fig. 10) and the small control nodule (Figs. 11 and 13), which were similar histologically. The result was a new, hybrid cancer, as one might call it, exhibiting the stigmata indicative of the virus action (Fig. 14).[4] The cells were considerably larger than those of either the parent tumor or the control nodule, and they increased still more in size as differentiation took place, ballooning instead of flattening and granulating prior to keratinization. The nuclei were also larger and as they enlarged further their chromatin marginated, coarse parakeratotic granules sometimes forming next them. The original tar tumor had been cystic, and the growing control implant was becoming so, but the cancers changed by the virus had almost completely lost this tendency (Fig. 12). The result of these changes was a carcinoma of comparatively coarse cytology, very active and highly aggressive. No such tumor has resulted from the intramuscular implantation of virus papilloma tissue in our numerous experiments of the sort; for though the papilloma, when gaining a foothold, may simulate cancer, as already mentioned, its epithelial layer soon rounds up into keratinized cysts like those of Fig. 3. The new, hybrid tumors of W. R. 100 E were evidently the outcome of the combined influence of the virus and of the principle actuating the original tar carcinoma, whatever that may have been.

The cancer to be next discussed grew actively at every control situation. There was thus introduced a difficulty which had not entered into the experiments with the benign tar tumors of domestic rabbits. In their case the consistent failure of the control implants to establish themselves brought with it a certainty that any proliferation of the material exposed to virus could be referred to the influence of the latter. But no such "open and shut" results could be expected with tumors capable of growing at the new situations without aid. Any stimulating effect of the virus upon them would find expression merely in relative rate of enlargement, and differences in this respect would diminish in proportion as the control grafts grew more rapidly. If the cells composing these were doing their utmost in the way of proliferation the virus could scarcely stimulate them further. And there was another complication,—the grafts exposed to virus inevitably contained cells which escaped contact with it because lying under the surface of the

[4] We have dealt with these stigmata in previous papers (*J. Exp. Med.*, 1936, 64, 401; 1940, 71, 469). No one of them is peculiar to tumors influenced by the papilloma virus, nor do such growths always show them, as the present work makes clear; yet when found together they are pathognomonic of the virus' action. A scrutiny of several hundred tar tumors of domestic rabbits and cottontails has failed to disclose any growth presenting the same association of cytological features, though many had a striking superficial similarity to virus papillomas.

tissue fragments. Hence some of the new tumors could not but be mixed growths, made up in part of the descendants of cells like those forming the control nodules. Whether such conditions obtained in the following instance cannot be said. But certain it is that the grafts exposed to virus grew no faster than did the controls, which enlarged with very great rapidity. Yet the virus "took" on the tar cancer cells, as proven by recovery of it from two of the implantation tumors, and by the hybrid morphology of one of them.

W. R. 1-47 E (Table II), tarred for not quite 4 months on both surfaces of the ears, had on the inside of the right an ulcerated, discoid tumor 1.4 cm. across and 4 mm. high, which had extended through to the outer side as a firm, subepidermal mound. It was nearly all taken for the experiment, but the remaining fragment enlarged during the 19 days before the rabbit was killed. The control material and that exposed to virus were implanted at six sites each. Abscesses developed at some of them and cancerous nodules at others. The virus had not evidently influenced the size or the morphology of the new tumors save at one situation where a new hybrid carcinoma nearly resembling that of W. R. 100 E was present, together with malignant tissue of the original sort. The recovery of virus will be considered further on.

In the remaining two instances the presence of virus papillomatosis in the implantation growths complicated the findings.

W. R. 1-40 E.—A large cancer was present on the outside of one ear of this animal, after they had been tarred for 4 months on both sides. It was 4 cm. across, 1.5 cm. high, raw and fungating, had extended through the cartilage and given rise to a big, ulcerated mound on the inner side. The ear was amputated and the neoplastic tissue next the cartilage was taken for the experiment. Section showed the growth to be a keratinizing, cystic, squamous cell carcinoma.

When the animal was killed 26 days after implantation, nothing was found at two of the control sites, abscesses at two more, with a small amount of surviving cancerous tissue at one of them, while at the remaining two, tiny nodules had formed of cancerous tissue like that of the original growth (Table II). Large tumors were present at all of the virus sites. One growth consisted of virus papillomatosis in the wall of an abscess, but four of the other five tumors consisted of cancer and papilloma intimately intermixed. The fifth tumor was composed of cancerous tissue like that of the original growth and of a new hybrid cancer nearly resembling that found in W. R. 100 E.

W. R. 61 N was tarred throughout two periods of many months each, at first on both sides of the ears. After 21 months in all, 3 months after the last tarring, the animal had an ulcerated growth 1.5 cm. across but only 4 mm. thick, projecting equally on each side of the ear. On the outer side several firm, blunt prongs extended toward the base of the organ in the subcutaneous tissue. The thickest part of the tumor was procured by punching, and the tissue next the cartilage was selected for the implantations. Section showed two distinct cancers in the material, one a keratinizing, cystic squamous cell carcinoma, the other having the same general character but with bizarre cells, many of them enormous and multinucleate. Embedded in the neoplastic tissue were numerous hair follicles, with melanoblasts amidst their cells.

The portion of the tumor left behind grew steadily during the 72 days before the animal was killed. At death it was 4.5 cm. across, thicker than before, and the extending prongs had broadened into a firm subcutaneous mass. Sections showed three distinct cancers, differing sharply in type, the third tumor being a squamous cell carcinoma with exceptionally small cells. It had doubtless been present in the implantation material as the results with this showed. There were no metastases.

Nodules had appeared rapidly at the four sites where material exposed to virus had been put, whereas at two of the control sites nothing could be found at autopsy, and, at the other two, tiny nodules (Table II). One of these proved to be a completely keratinized cyst (Fig. 16), the other a little cyst walled with degenerating cancerous tissue and containing debris (Fig. 15). The implantation growths from the material exposed to virus were by comparison of great size, from 1.3 to 5 cm. in diameter. One consisted wholly of virus papilloma while the others were mixtures of cancer and virus papilloma in varying amounts, the latter being almost absent from one of two axillary nodules (Table II). No hybrid cancer had developed but all three types of malignancy found in the original growth at autopsy were represented (Figs. 18 to 20); and at most situations the papilloma tissue had formed separate, discrete nodules consisting of the usual creamy or gray cysts enclosing keratinized epithelium (Fig. 17). The separation of papilloma and cancer enabled us to make comparative tests for the presence of virus in the two sorts of tissue.

The implantation growths developing in W. R. 1-40 E from material exposed to the virus were close-textured and appeared entirely cancerous in the gross, but the microscope showed in most instances a mixture with virus papilloma tissue. The cancer was largely of the original type, with a new hybrid carcinoma at some situations. One nodule consisted wholly of the two. In W. R. 61 N the virus papilloma tissue had the form of discrete nodules. They were largely melanotic, a condition which pointed to an origin from the hair follicles present in the implanted fragments of the tar tumor. This latter contained no melanoblasts.

The virus stimulated the cancer cells in both these instances, and in one altered them morphologically.

The Recovery of Virus from Implantation Growths

Virus of high pathogenicity, such as we employed, produces vigorous papillomas in domestic rabbits, but from them it can only exceptionally be got again; and it has never been procured from the derivative cancers. In view of these negative findings we made no attempt in the present work to recover virus from the tumors due to the implantations in domestic rabbits.[5]

[5] By testing many cottontail papillomas Shope procured some strains of the virus which produced in domestic rabbits growths from which it could be recovered (*Proc. Soc. Exp. Biol. and Med.*, 1935, **32**, 830). The strains gave rise only to indolent papillomas, after a long incubation period, and hence were unsuited to the present work.

The conditions are different in cottontails. Virus can be recovered in abundance from their papillomas when these are not so disordered that neutralizing antibody from the blood extravasates into them (24). This quite frequently happens, however, with result that no virus can be got from the growths; and so pronounced is the extravasation of antibody into the derivative cancers,—which regularly fail to yield virus,—that often extracts of the malignant tissue prove capable of neutralizing the latter in considerable quantity *in vitro* (25). None of the antibody is present in animals carrying tar cancers (16), and it appears gradually when virus papillomas are produced in normal rabbits (26). In view of these facts there seemed to be a possibility that virus would be obtainable from some of the malignant tumors resulting from the implantations in cottontails, if the animals were killed early. This has proved to be the case. Unfortunately no tests were made in the instance which provided the most favorable conditions,—that of W. R. 100 E, which developed large cancers within 2 weeks after implantation.

The implantation tumors were removed with due precautions for asepsis, and cut up and placed in 50 per cent glycerin, after slices of them had been taken in Zenker's fluid. Virus papilloma tissue and cancer tissue lying separate in the same mass were handled with different instruments and glycerinated separately. A few weeks later 10 per cent Tyrode extracts were made of the materials and inoculated into checkerboard squares on each of three normal domestic rabbits, which were bandaged afterwards in such way as to prevent any transfer of the inocula (26). The following symbols are employed in Table II to report the findings: ++++ = confluent papillomatosis, +++ = semiconfluent papillomatosis, ++ = many discrete papillomas, + = a few papillomas, ± = 2, 3, or 4 discrete papillomas, ± = 1 papilloma. The results of the inoculations were recorded at frequent intervals until 35 days had elapsed, after which no more growths appeared.

One of the implantation cancers of W. R. 1-47 E,—killed after 19 days,—yielded no virus; but from two others a little was got. The papilloma on the side yielded scarcely more, and the tumors deriving from the control implants gave none. One of the growths from which virus was obtained had a hybrid morphology indicative of its influence. No tests were made to learn whether antibody had appeared in the blood of the animal, as sometimes happens even so early.

The blood of W. R. 1-40 E, killed 26 days after implantation, contained antibody in considerable amount, as disclosed by complement fixation tests. Only one of the tumors was wholly cancerous. It yielded virus, but merely a trace as compared with the abundance obtained from the papilloma on the side of the animal. Most of the growths consisted of mixtures of cancer and virus papilloma tissue, and they yielded virus in amounts roughly corresponding to the proportion of the latter. In one growth the two were so sharply separated that both could be tested; and both yielded virus, more coming from the papilloma tissue. Only one of the control implants provided sufficient material for test. No virus was obtained from it.

The findings with W. R. 61 N were enlightening in several respects. The inoculation

growth on the side yielded only a moderate amount of virus, one of the large implantation growths which consisted wholly of virus papilloma tissue yielded still less, and none at all could be got from the discrete, virus papilloma nodules forming part of another large implantation mass which consisted for the rest of cancer (Fig. 17). This latter gave no virus. A little was got from another tumor consisting mostly of cancer but with some virus papilloma tissue intermixed. None of the control implants provided enough tissue for test, but the original tar cancer on the ear, which had continued to enlarge, failed to yield virus. Serum tests were not made, but the rabbit had carried large papillomatous masses for more than 7 weeks and the likelihood is that its blood contained antibody in considerable amount.

The results with the growths of W. R. 1-40 E and W. R. 61 N show that papillomas lying deep are less likely to yield virus in quantity than are those on the skin surface, local circumstances altering cases much in this respect. From one of the actively growing intramuscular papillomas of W. R. 61 N no virus whatever could be procured, although it was got from another implantation tumor in which cancer predominated. In general the cancers yielded far less virus than did the associated papillomatous tissue. The reasons will be discussed further on.

Implantation of a Tar Carcinoma of the Domestic Rabbit

The tar we employed does not elicit cancer in domestic rabbits until after many months have gone by, and only one growth of the sort was available for implantation tests.

The cancer animal had been tarred throughout three periods, totaling 5 months, with long intervals between them. After 21 months in all, 6 months after the last tarring, a persisting papilloma was noted to have ulcerated, and a gland at the base of the ear was found to be large and firm. During the next month the tumor grew with great rapidity, destroying part of the ear, and numerous large, firm nodules developed under the jaw and further down the neck in the lymph glands. The enlarged gland at the base of the ear was now excised, and the neoplastic tissue which had almost entirely replaced it was hashed, exposed to virus, and implanted in the usual way. When the animal died 58 days later, abscesses had developed in some of the metastases along the neck and others had coalesced into huge masses. Nothing was found at one of the situations where cancer tissue unexposed to virus had been placed, but at the other four large tumors were present, as also at all five spots where the material exposed to virus had been put, some of the growths being 5 cm. in diameter. There had been no significant difference in their rate of enlargement. The original tar tumor was an anaplastic, squamous cell carcinoma, very unhealthy and necrotizing early.

In this instance vigorous papillomas appeared where the skin had been inoculated, yet the virus caused no morphological alteration or stimulation of the cancerous tissue exposed to it.

DISCUSSION

The Findings in Domestic Rabbits

In a previous paper we have reported that the benign, epidermal tumors elicited by tarring the skin of domestic rabbits are wholly conditional neoplasms, dependent for their persistence either on continued tarring, on the chronic changes this eventually brings about in the supporting tissues, or on inflammation and maceration incidental to crowding, when the growths are large and many (2, 19). The present results accord with these findings. Not one of 57 benign but vigorous tar tumors transplanted within the host to situations well suited to the growth of implanted cancers and virus papillomas was able to establish itself. The tumor cells actually proved less capable in this respect than the normal cells accidentally transferred with them. Living hair follicles in good condition, sebaceous glands and minute cysts lined with ordinary squamous epithelium were not infrequently met at sites from which all neoplastic elements had disappeared.

The hyperplastic epidermis punched from places on the tarred ears where no tumors were visible and implanted after exposure to the virus regularly yielded nodules of virus papilloma tissue. No "anomalous tumors" developed such as arise fairly often when the virus localizes in areas on tarred ears which are devoid of growths (2); but the conditions of the present experiments were not favorable to their occurrence. Even when the virus localizes abundantly in the ears and gives rise to innumerable papillomas of typical sort where previously there was only hyperplasia, the anomalous growths are never many, whence one can conclude that the cells capable of forming them must be relatively few. The chances were greatly against the exposure of such cells to virus infection when the skin was cut into half-millimeter pieces—the average size of the hashed fragments employed in the experiments.

On many of the tar tumors the virus seemed to have no stimulating effect, these failing to grow at any implantation site. This cannot have been due to lack of pathogenicity of the inoculum since it regularly acted upon fragments of the tarred skin of the same animals. Destruction of the grafts by bacterial infection can be invoked to explain some failures, notably in the case of tissue procured from ulcerated carcinomatoids,—which as a group gave a high proportion of negative results,—but this will not cover the findings with orderly, "healthy looking" growths. At not a few sites aggregates of keratinized scales showed that the graft had died by differentiation, while at others tiny cysts remained, lined with stratified squamous epithelium. In these instances bacterial action cannot have

caused the failures. We have repeatedly noted that some of the tar tumors which were present on the ears at the time when the virus localized in these organs behave as if wholly uninfluenced by it, altering not at all or disappearing while everywhere else over the entire surface an active, crowded, virus-induced proliferation is going on (2). Several of the cottontail cancers of the present work, which grew after implantation, were not perceptibly altered by the virus. One must conclude that the cells of certain tar tumors are refractory to its influence. Whether they become infected with it is uncertain.

The positive yield of the implantations was small as compared with that when the virus takes effect in benign tar tumors situated on the ears. There the conditions favor its association with cells already neoplastic,—a fact clearly evident in the frequency with which it affects the tar tumors when it localizes at only a few spots,—and virus papillomatosis obscures its stimulating and altering influence far less often than when pieces of tar tumors, containing normal as well as neoplastic cells, are submitted to its action. However carefully the fragments are trimmed, normal elements can seldom be excluded from them; for non-neoplastic epidermis is often present in the clefts between the fingers of papillomas, and hair follicles extend down both into them and the carcinomatoids. A considerable proportion of the papilloma nodules arising on implantation may have derived from such components. Sometimes though, a conversion of tar papilloma cells into virus papilloma cells may have occurred; for the change has been repeatedly noted in sections of tar tumors removed from the ears shortly after the localization of intravenously injected virus. In whatever way the new virus papilloma tissue arose, it rapidly outgrew any surviving tar tumor tissue, and soon so preponderated as to hide the latter.

A few of the tar papillomas retained their characteristic morphology though impelled to grow by the virus. The possibility has been ruled out by previous work (2) that the carcinogenic effect of the latter upon tar tumors is due merely to conversion of the growths into virus papillomas, with malignant changes so rapidly ensuing in the latter as to be telescoped into a few days instead of taking place after months or years, as under ordinary circumstances (27). The results of the implantations exclude this possibility also. One can readily tell where virus papilloma has been present in muscle or connective tissue, since on dying it leaves laminated, keratinized pearls of distinctive form, which are only very slowly resorbed. Microscopic search of the cancers which arose at spots where carcinomatoids infected with the virus had been implanted yielded no such evidence that they had been preceded by virus papillomatosis.

Many of the growths which result from the changes taking place in tar tumors after virus has localized in the ears have no duplicates amongst either the tar or virus tumors but exhibit a blend of their characters. They are the anomalous tumors already referred to. The implantations in domestic rabbits did not yield any of these hybrid or mongrel growths,—a fact scarcely surprising in view of how few tumors, other than virus papillomas, were obtained in all; but several hybrid growths arose from the implanted fragments of cottontail cancers that had been exposed to virus infection. They will be discussed further on. Only the carcinomatoids of domestic rabbits, not the tar papillomas, grew as cancers after implantation, but this is not expressive of a rule since we have repeatedly seen tar papillomas on the ears undergo alteration to rapidly growing cancers as result of infection with the virus (2),—a transformation followed by repeated biopsies. Yet it was also noted that carcinomatoids are more likely to be rendered malignant in this way. The possibility has to be considered in such relation that the carcinomatoids which become actively cancerous after infection with the virus are actually ineffective carcinomas, incapable of maintaining themselves without aid. Yet since the carcinomatoids as a group have proved to be mere phases of tar papillomatosis (19), and since tar papillomas themselves not infrequently become cancerous under the influence of the virus, there would seem to be no reason to assume the existence in domestic rabbits of an additional category of benign growths. The carcinomatoids obviously have cells far more sensitive to the stimulus of tarring than those of tumors of the same essential character which retain the papilloma form. They mimic true cancers as the latter do not though submitted to the same conditions (19). At the time when the papilloma virus infects their cells these are already invading and destroying and are more or less anaplastic. All that the virus need do to render them truly cancerous is to assure their continued activity in the state they have already assumed, in the same way that it assures the progressive growth of certain tar papillomas though causing no morphological alterations in them. Whatever the fact, the statement of a previous paper (14) that "one cannot suppose anaplastic squamous cell carcinomatosis in either rabbits or man to be maintained by agents that act as adjuvants to the essential cause of the neoplastic condition" must be withdrawn.

Many of the tar papillomas of mice yield cancers at once when stimulated by injury, or cut up and implanted (28). The malignant growths then arising may be the outcome of secondary changes in the papilloma cells brought about by injury,—which frequently precipitates a cancerous change in virus papillomas (27),—or they may conceivably be due to the liberation

of malignant cells already present in the apparently benign growths. Our implantation experiments with rabbit papillomas which had long been tarred or had long persisted, were undertaken as bearing upon these alternatives. They yielded no evidence of any increased capacity of the papillomas to grow on implantation, nor of the existence of latent cancer cells. Yet needless to say, such cells may be present in the growths later on, since most of the tar carcinomas of rabbits arise from preëxisting papillomas.

The Findings in Cottontail Rabbits

The virus failed to influence some of the cottontail cancers perceptibly, just as it failed with some of the benign tar tumors of domestic rabbits. Other cancers it impelled to grow rapidly, often altering their form. It did not stimulate those that were inherently capable of growing at a great rate, though sometimes it altered them.

The actuating factor in cottontail cancers, whatever this may be, causes them to proliferate at differing rates. Other things being equal, one would expect the stimulating effect of the virus to be most evident upon slow-growing tumors; and this was the experimental finding. In two instances (W. R. 100 E, 61 N—Table II) the virus was the factor determining success at the new sites, the control grafts doing practically nothing. Its inability to hasten the enlargement of cancers capable of growing quickly without its aid can be explained by assuming that the malignant cells were already urged to their maximum. This would cover the case of the tar carcinoma of a domestic rabbit which the virus failed to influence. It was certainly present in the implantation nodules of one of the fast-growing cottontail cancers (W. R. 1-47 E) which gave no sign of stimulation, for they not only had a morphology indicative of its presence but yielded it on extraction.

Very little virus was recovered from the cancerous implants, considering the great changes that it induced in them (Table II). More was usually got from tumors containing some virus papilloma tissue, and still more from growths consisting wholly of the latter. Yet even from these less virus was procured than from papillomas produced on the skin of the animal by the same inoculum. No virus whatever was obtained from one of the papilloma nodules of W. R. 61 N, though another yielded it (Table II). The observed differences were doubtless due mostly to differences in the local conditions affecting the extravasation into the growths of virus-neutralizing antibody; for such extravasation often wholly prevents the recovery of the virus from skin growths known to contain it (24). Cancers ordinarily provide conditions more favorable to extravasation than do papillomas (25), and hence one would expect them to yield less virus.

But a possibility exists that even the small amount obtained from the cancerous implantation tumors really came from virus papilloma tissue situated in parts of the growths which could not be submitted to microscopic scrutiny because extracted for test. The best evidence for the presence of the virus in the implantation cancers lay in their altered character.

General Considerations

On reviewing the results as a whole one perceives that the effects of the virus on the implanted tar tumors fell into five categories:

1. In many instances it changed the tumors to virus papillomas.

2. It brought about the formation of hybrid tumors, morphologically expressive of the combined influence of the virus and of the unknown factor responsible for the neoplastic state of the tar tumor cells. The hybrid condition was sometimes, though not always, accompanied by an increased rate of tumor growth.

3. It converted some previously benign tumors into cancers showing no morphological sign of its presence.

4. It enabled some tumors to establish themselves and grow, while retaining their characteristic features, and it hastened the proliferation of others capable of establishing themselves without its aid.

5. It failed in some instances to influence the tumor cells perceptibly.

These findings corroborate and extend observations made upon tar tumors *in situ* (2). The influence of the virus ranges all the way from apparently complete domination of the tar tumor cells, with result that they become virus papilloma cells, to equally complete ineffectiveness. Often, as in the anomalous tumors several times mentioned, the alterations in the neoplasm appear to be the expression of some balance of power struck with the intrinsic cause or causes for the tar tumors. Judging from the behavior of the latter these causes are generally less forceful than the virus. Hence, perhaps, the frequency with which it becomes the dominating influence.

Our recent work with the virus has nearly all been directed to the problem of its relation to the carcinomas originating from the papillomas it engenders. Are they due to some other cause than the virus, acting upon cells which the latter has prepared? Or does the virus persist in them and, though not their cause, exert a modifying effect upon them? Or is the virus, or a variant of it, the actuating principle in the malignancy?

Something can now be said on these matters. The presence of the virus has been demonstrated in two cancers deriving from the papillomas experimentally induced with it in domestic rabbits (15, 29). One of the growths

has been transplanted to thirteen successive groups of animals thus far, and serological tests have shown that the virus regularly persists in the tumors and increases in amount as they enlarge in the new hosts.[6] The present work proves, like experiments already reported (2), that the virus is able to influence tumors due to some cause unrelated to it antigenically (15), namely tar papillomas and carcinomas. It has proved itself capable of goading these to more rapid proliferation, of altering their form and rendering some cancerous when otherwise they would have remained benign and eventually have disappeared. In initiating and maintaining malignancy in benign tar tumors it acts as the effective cause for cancer. Since it can stimulate and alter some of the squamous cell carcinomas elicited with tar in its native host, the cottontail rabbit, there is every reason to suppose that it can influence the cancers of similar histological sort, derived from cells that it has itself rendered papillomatous. Actually there are signs of its influence in many such cancers (27).

Yet one cannot conclude that the virus is more than an adjuvant influence in the malignant growths. It would seem to be merely this in the tar tumors, though frequently decisive for their character and fate.[7] None of a hundred and more virus materials provided by the naturally occurring papillomas of cottontails has ever on test given rise to anything but benign papillomas of identical morphology, when rubbed into scarified normal epidermis. To function as the cause for the carcinomas deriving from such growths it must either have undergone variation, with result that it expresses itself in altered cellular ways, or else the conditions of its partnership with the cell,—a partnership which ordinarily results in neoplastic growths of stereotyped character,—must have changed, or the state of the cell which is its medium of expression must have altered (2). In recent papers we have brought evidence favoring the view that the malignancy is due to virus variation (30, 25).

The demonstration that the virus may exert driving and formative effects upon cells rendered neoplastic by another means brings up the possibility that under natural conditions intercurrent infection with viruses may sometimes stimulate and alter tumor cells which otherwise would never disclose themselves. The influence of bacterial infection to enhance the malignancy

[6] This finding can scarcely be deemed remarkable in view of the ability of virus papilloma cells and those of the derivative cancers to support a multiple infection with wholly alien viruses (Syverton, J. T., and Berry, G. P., *Am. J. Path.*, 1938, **14**, 633).

[7] The virus recovered from the implanted cottontail cancers of the present work gave rise only to papillomas on inoculation: it had not become a directly carcinogenic virus in its influence upon scarified normal epithelium.

of human tumors has long been recognized, though its scope is still undetermined. Rivers and Pearce's discovery that virus III had entered the Brown-Pearce rabbit tumor they were working with, and flourished under the circumstances of further transplantation, sufficiently shows that an extraneous virus can reach and infect cancers.[8] But the literature reviewed at the beginning of the present paper indicates that viruses which exert no proliferative influence upon cells under ordinary circumstances have none upon tumors into which they are introduced. Furthermore the neoplastic viruses thus far studied have proved to be remarkably selective in their action. Each is pathogenic only for species nearly related to that from which it came, and each causes cells of but a single sort to become tumor cells. The rabbit papilloma virus will not persist on introduction into the Brown-Pearce rabbit carcinoma (16), a growth presumably originating in a hair follicle (31), and inducing no immunity against the virus. The virus of chicken tumor I, which causes sarcomas when injected into connective tissue, is devoid of influence upon the sarcomas of connective tissue origin elicited with chemical carcinogens, and soon disappears from them (11). All this speaks for the great rarity of secondary virus infection as a precipitating cause for tumor growth under natural conditions, if indeed it ever acts in such a way.[9]

In some recent experiments bits of tar cancers from cottontail rabbits were exposed *in vitro* to Tyrode and to the Shope fibroma virus (32) respectively, with implantation afterwards in the leg muscles and subcutaneous tissue of the host. The general procedure was that used in the work with the papilloma virus, and the proliferative strain of virus (OA strain of Andrewes and Shope (33)) was employed. Its effects were tested upon two or three cancers of each animal. It gave rise to characteristic fibromas where the fragments exposed to it had been implanted, and the cancerous tissue included in the growths proliferated more actively in some instances and with more anaplasia

[8] Andrewes (*J. Path. and Bact.*, 1940, **50**, 227) has recently encountered virus III as a damaging contaminant in infectious fibromas of rabbits, growths due to another virus. In addition to producing inclusion bodies, the virus III sometimes caused focal necroses and early retrogression of the fibroma. Andrewes found it also in a repeatedly transplanted sarcoma which had arisen where an infectious fibroma had been present.

[9] McIntosh has lately reported that a strain of the virus of contagious dermatitis of sheep, maintained in rabbits for 3 years and producing proliferative cutaneous lesions of the skin of such animals, failed to affect tar tumors when injected intravenously (McIntosh, J., *16th Ann. Rep. Brit. Empire Cancer Campaign*, London, 1939, 33). He has confirmed our finding that the Shope papilloma virus, thus introduced, causes such tumors to grow more rapidly and in many cases to become malignant. Brunschwig, Tschetter, and Hamann tried to induce cancer in rabbit ears previously treated with benzpyrene, by injecting extracts of human warts intravenously. The results of their experiments were equivocal (*Am. J. Cancer*, 1940, **38**, 50).

than that of the control implants. The difference might have been great had not most of the cancers exerted a strong desmoplastic influence of their own. Some of them evoked at the control situations a connective tissue reaction almost as lively as that due to the virus, though differing in detail; and it was obviously favorable to growth of the tumor.

The carcinoma cells proliferating amidst the fibroma tissue frequently showed the eosinophilic granulation which Shope and others have described (32) as occurring in the epidermis overlying superficial fibromas produced with the virus. Ordinarily these latter retrogress in cottontails after some weeks, and they do so even sooner in domestic rabbits, though tar injections will cause them to enlarge for a much longer time, sometimes with secondary dissemination (12). It had been hoped that the months of preliminary tarring whereby the cancers had been evoked in our implanted cottontails might have so shaped conditions that the fibromas would grow progressively; but this was not the case. Retrogression began within the ordinary period.

The interpretation of the phenomena occurring when the papilloma virus localizes in tarred ears will be the theme of a later paper.

SUMMARY

The effects of the rabbit papilloma virus upon tar tumor tissue are widely various, as the present paper and previous ones attest. It enables some of the benign tar tumors of domestic rabbits (papillomas, carcinomatoids) to establish themselves after implantation,—which they are unable to do under ordinary circumstances, being dependent upon favoring factors; and it may drive them to active proliferation without altering their morphology. Some growths it fails to influence and some it converts into virus papillomas. Often, however, it brings about cytological changes which are indicative of a combination of its influence with that of the undetermined factor motivating the original tumor. The resulting neoplasm exhibits a blend of characteristics.

The virus makes some benign tar tumors become cancerous forthwith, the malignancy developing without intermediate virus papillomatosis. It can be readily imposed upon some of the squamous cell carcinomas which tar elicits in its natural host, the cottontail rabbit, and it may drive such tumors to proliferate faster, or alter them morphologically, or do both. Its stimulating effect is especially pronounced in the case of those tar cancers that are slow-growing.

Since the virus can influence tar cancers markedly, one can scarcely suppose it to be devoid of effect upon the cancers of the same type which derive from the papillomas it has itself engendered. Other implications of the work are discussed.

BIBLIOGRAPHY

1. Shope, R. E., *J. Exp. Med.*, 1933, **58**, 607.
2. Rous, P., and Kidd, J. G., *J. Exp. Med.*, 1938, **67**, 399; **68**, 529.
3. Levaditi, C., and Nicolau, S., *Compt. rend. Soc. biol.*, 1922, **87**, 498.
4. Levaditi, C., and Nicolau, S., *Ann. Inst. Pasteur*, 1923, **37**, 443.
5. Levaditi, C., Schoen, R., and Reinié, L., *Ann. Inst. Pasteur*, 1937, **58**, 353.
6. Levaditi, C., and Haber, P., *Compt. rend. Acad. sc.*, 1936, **202**, 2018. Levaditi, C., Schoen, R., and Reinié, L., *Compt. rend. Soc. biol.*, 1937, **124**, 711. Levaditi, C., and Schoen, R., *Compt. rend. Soc. biol.*, 1937, **125**, 607.
7. Schoen, R., *Compt. rend. Soc. biol.*, 1938, **128**, 135; *Ann. Inst. Pasteur*, 1938, **60**, 499.
8. Findlay, G. M., and MacCallum, F. O., *Tr. Roy. Soc. Trop. Med. and Hyg.*, 1937, **30**, 507.
9. Pownall and Florey, H. W., *12th Ann. Rep. Brit. Empire Cancer Campaign*, London, 1935, 105.
10. Rivers, T. M., and Pearce, L., *J. Exp. Med.*, 1925, **42**, 523.
11. Mellanby, E., *12th Ann. Rep. Brit. Empire Cancer Campaign*, London, 1935, 99; *J. Path. and Bact.*, 1938, **46**, 447.
12. Andrewes, C. H., and Ahlström, C. G., *J. Path. and Bact.*, 1938, **47**, 87.
13. Andrewes, C. H., Ahlström, C. G., Foulds, L., and Gye, W. E., *Lancet*, 1937, **2**, 893.
14. Rous, P., Beard, J. W., and Kidd, J. G., *J. Exp. Med.*, 1936, **64**, 401.
15. Kidd, J. G., Beard, J. W., and Rous, P., *J. Exp. Med.*, 1936, **64**, 79. Kidd, J. G., and Rous, P., *J. Exp. Med.*, 1940, **71**, 813.
16. Kidd, J. G., Beard, J. W., and Rous, P., *J. Exp. Med.*, 1936, **64**, 79.
17. Levaditi, C., and Schoen, R., *Compt. rend. Soc. biol.*, 1936, **122**, 736.
18. Syverton, J. T., and Berry, G. P., *Am. J. Path.*, 1938, **14**, 633.
19. Rous, P., and Kidd, J. G., *J. Exp. Med.*, 1939, **69**, 399.
20. Borst, M., *Z. Krebsforsch.*, 1924, **21**, 341.
21. Ferrero, V., *Arch. sc. med.*, 1926, **48**, 67.
22. Rous, P., and Beard, J. W., *J. Exp. Med.*, 1934, **60**, 701, 723, 741.
23. Rous, P., Kidd, J. G., and Beard, J. W., *J. Exp. Med.*, 1936, **64**, 385.
24. Kidd, J. G., *J. Exp. Med.*, 1939, **70**, 583.
25. Kidd, J. G., and Rous, P., *J. Exp. Med.*, 1940, **71**, 469.
26. Kidd, J. G., Beard, J. W., and Rous, P., *J. Exp. Med.*, 1936, **64**, 63.
27. Rous, P., and Beard, J. W., *J. Exp. Med.*, 1935, **62**, 523.
28. Cramer, W., *Brit. J. Exp. Path.*, 1929, **10**, 335. Mottram, J. C., *Am. J. Cancer*, 1934, **22**, 801; *J. Path. and Bact.*, 1936, **42**, 79.
29. Kidd, J. G., and Rous, P., *J. Exp. Med.*, 1940, **71**, 813.
30. Rous, P., *Am. J. Cancer*, 1936, **28**, 233.
31. Brown, W. H., and Pearce, L., *J. Exp. Med.*, 1923, **37**, 601.
32. Shope, R. E., *J. Exp. Med.*, 1932, **56**, 793, 803.
33. Andrewes, C. H., *J. Exp. Med.*, 1936, **63**, 157. Shope, R. E., *J. Exp. Med.*, 1936, **63**, 173. Andrewes, C. H., and Shope, R. E., *J. Exp. Med.*, 1936, **63**, 179.

EXPLANATION OF PLATES

All of the sections were stained with eosin and methylene blue.

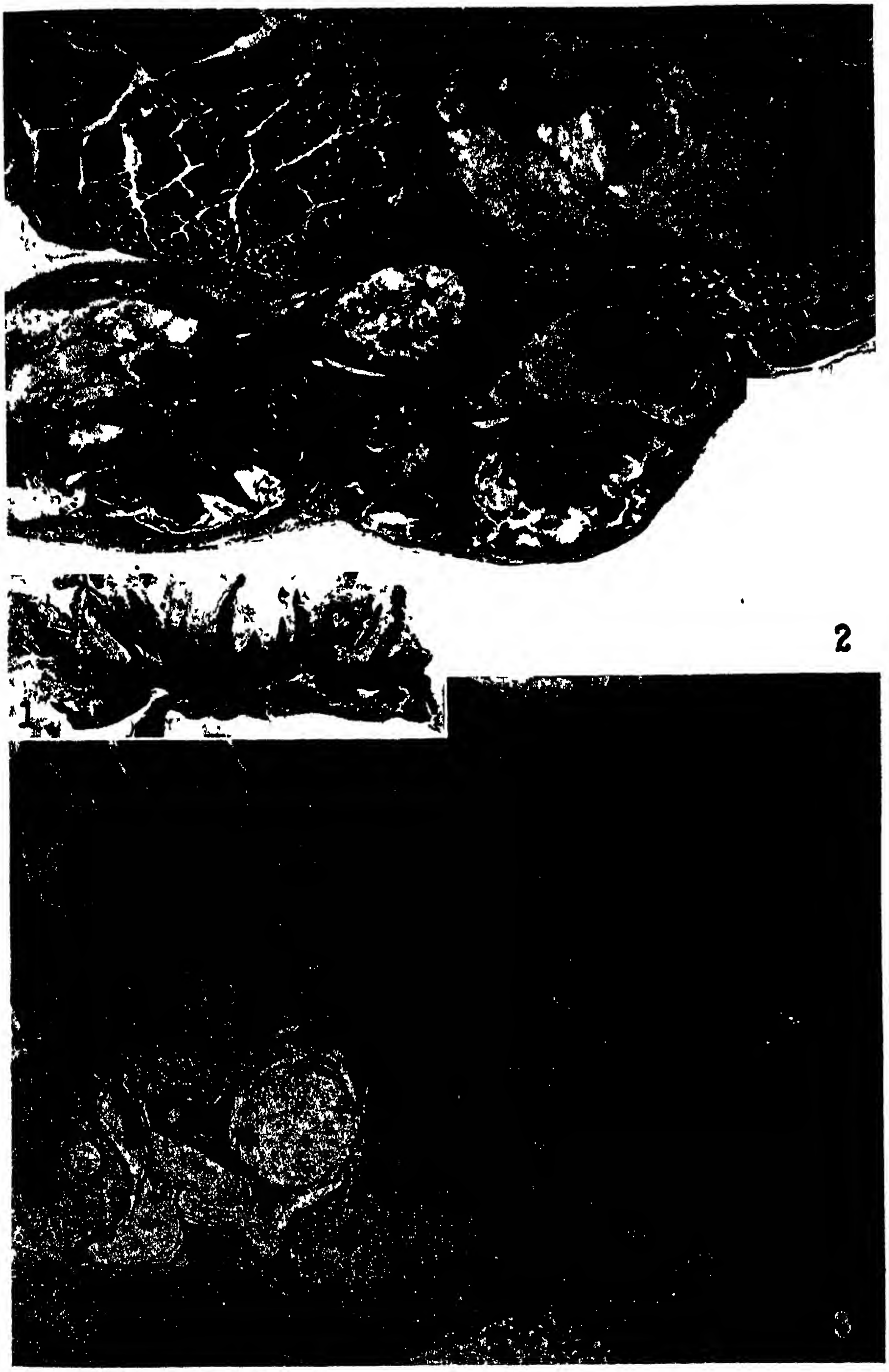
Plate 36

Figs. 1 and 2. Effects of the Virus on a Tar Papilloma of the Domestic Rabbit.—

Fig. 1. Cross-section of a growth excised from the ear of D. R. 3-73. × 12.

Fig. 2. Results of implanting in the leg muscle bits of the tumor of Fig. 1, which had been exposed to the virus. Each fragment has given rise to a growth with the morphology of the original tumor. The proliferating epithelium has rounded into knobs connected with the periphery by connective tissue pedicles, and keratinization has taken place *outwards*. Animal killed on the 38th day. No trace was found of the tumor bits used for the control implantations. × 12.

Fig. 3. For comparison with Fig. 2. Part of a nodule consisting of virus papilloma tissue, which resulted from the intramuscular implantation of bits of another tar papilloma exposed to virus. The epithelium has rounded into cysts and is keratinizing *inwards*, as always with virus papillomas under such conditions. × 12.



Photographed by Joseph B Haulenbeek

(Rous and Kidd Effects of papilloma virus upon implanted tar tumors)

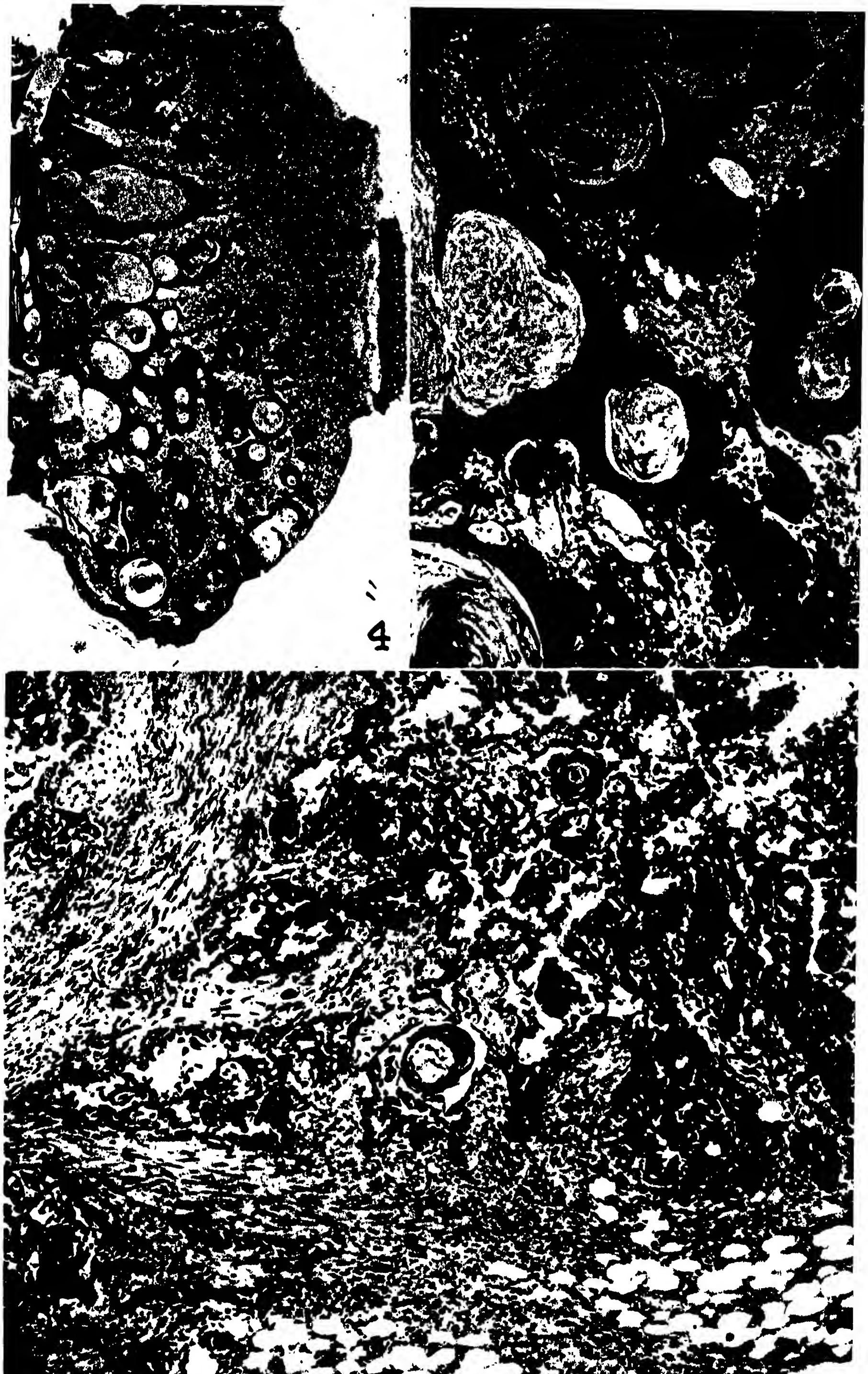
Plate 37

Effects of the Virus on a Tar Carcinomatoid of the Domestic Rabbit.—

Fig. 4. Cross-section of the piece of tumor removed from the ear of D. R. 4-97. About half of the growth was left behind, and this disappeared in the course of the 20 days before the animal died. Tarring of the other ear had been continued. × 20.

Fig. 5. Part of the specimen at a higher magnification. It looks like a squamous cell carcinoma. × 80.

Fig. 6. Periphery of the nodule, 1.5 cm. in diameter, which resulted from implantation in the subcutaneous tissue of fragments of the tumor, which had been exposed to the papilloma virus. The growth is actively malignant and more anaplastic than the original carcinomatoid. At the lower left-hand corner of the photograph its cells can be seen invading the fatty tissue. There is no sign that it has been altered by the virus in the direction of papillomatosis. The control fragments had completely vanished. × 80.

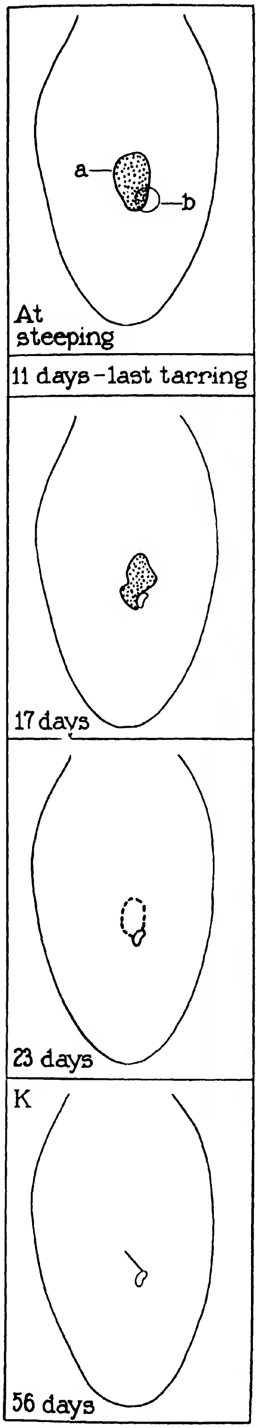


Photographed by Joseph B. Haulenbeek

Plate 38

Effects of the Virus on a Tar Carcinomatoid of D. R. 5-10.—

Figs. 7 to 9. The growth (*a*) was a raised, raw disc, 2.3 cm. across, when a small part of it was punched out, as indicated by the circle in the outline drawing. Some of the near-by tissue was removed incidentally. No tarring had been done for 10 days and the neoplastic tissue was rounding up into cysts. Fig. 7 ($\times$ 6) shows in cross-section the tumor material procured. It was cut fine and implanted in the usual way, tarring was resumed for 11 days more, and on the 56th day the animal was killed. The carcinomatoid was gone by then, leaving a mound covered with smooth epidermis next the small hole in the ear. A slice was taken through this mound, as indicated by the heavy line in the lowest drawing. Sections showed it to consist entirely of connective tissue save at one small spot (arrow) where were dying carcinomatoid cells surrounded by foreign body giant cells (Fig. 8; $\times$ 5). The overlying epidermis was devoid of downgrowths. Nothing was left where the control fragments of tumor had been implanted. Those exposed to virus, on the other hand, had given rise to an actively invasive, multicentric cancer, 2 cm. across (Fig. 9; $\times$ 30).

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Photographed by Joseph B. Haulenbeek

(Rous and Kidd: Effects of papilloma virus upon implanted tar tumors)

PLATE 39

Effect of the Virus on a Tar Carcinoma of Cottontail Rabbit W. R. 100 E (Table II).—

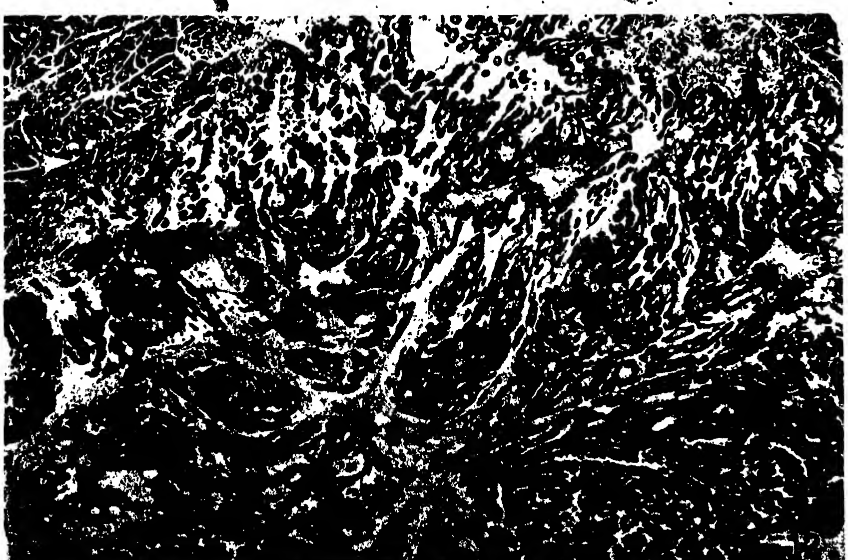
FIG. 10. Section of the biopsy specimen utilized for the test. The growth is a cystic, squamous cell carcinoma of orderly character. × 18.

FIG. 11. Cross-section of the only nodule found at any of the five situations where control fragments of the growth had been implanted. It was 0.2 cm. across. All except one of the bits of tumor tissue are dying or dead, after having formed keratinized cysts. This one has started to grow, and the tongues of neoplastic cells have begun to form cysts with a thick outer layer of living epithelium, like those in the original neoplasm. (For higher magnification see Fig. 13.) × 14.

FIG. 12. Part of one of the large growths found where the cancer fragments exposed to virus had been put. It is a very aggressive carcinoma, wholly unlike the original tar tumor. (See Fig. 14.) × 14.

10

11



Photographed by Joseph B. Haulenbeck

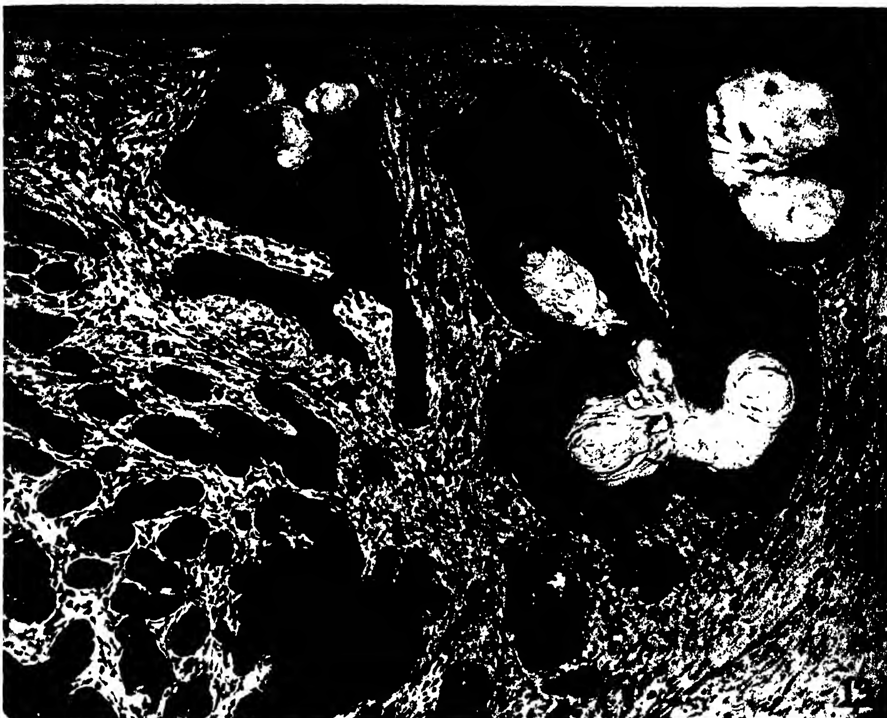
(Rous and Kidd: Effects of papilloma virus upon implanted tar tumors)

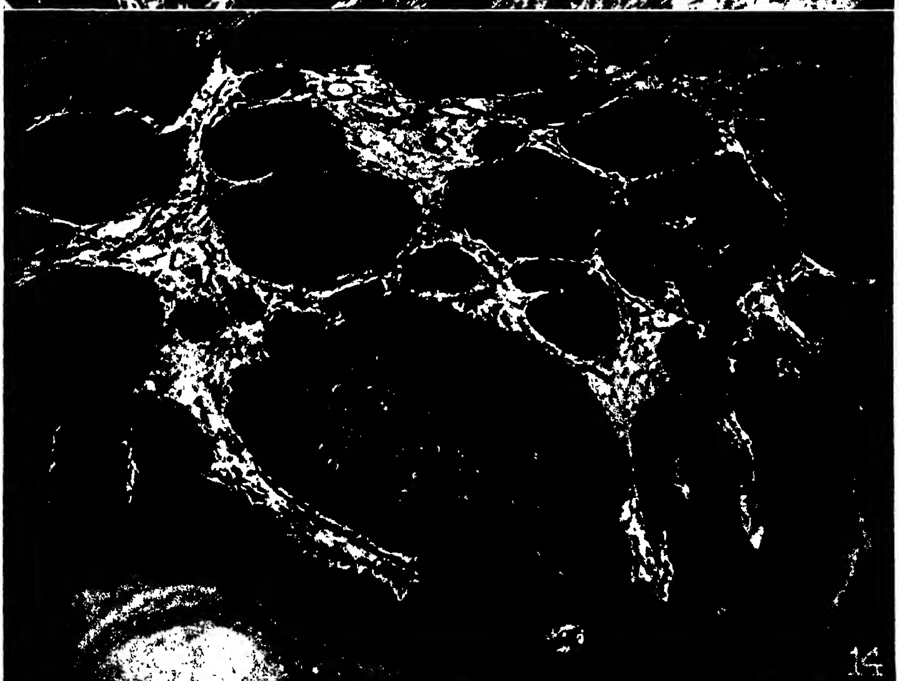
PLATE 40

The implantation growths of Figs. 11 and 12 at high magnification,—to show the alterations brought about by the virus.

FIG. 13. Growing portion of the control nodule of Fig. 11. The tumor resembles the original cancer cytologically. $\times$ 95.

FIG. 14. The implantation cancer of Fig. 12. The tongues of carcinomatous tissue are much coarser than those in the control nodule, and the cells are larger and exhibit stigmata indicative of the influence of the virus. $\times$ 95.





Photographed by Joseph B. Haulenbeek

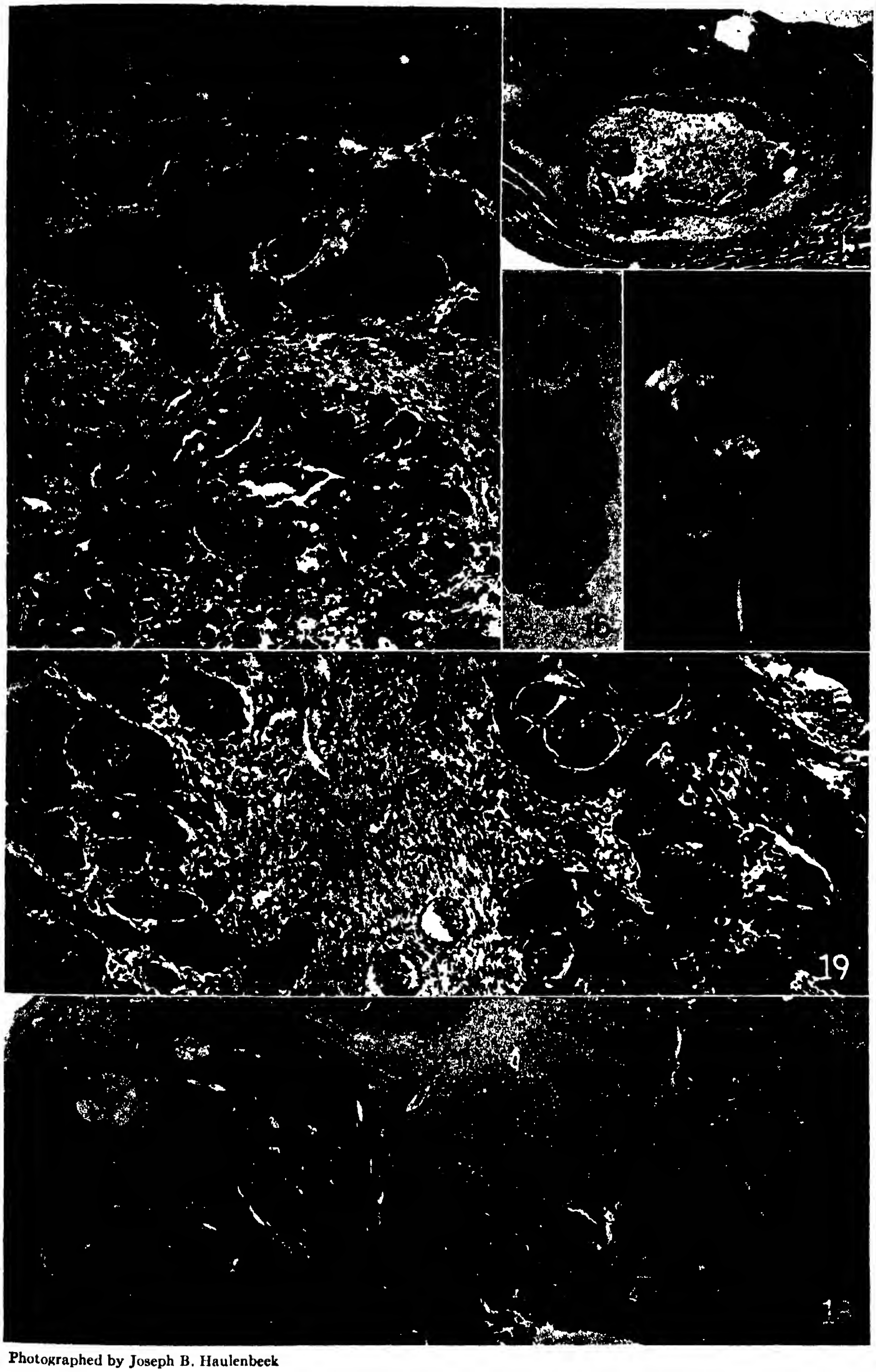
(Rous and Kidd: Effects of papilloma virus upon implanted tar tumors)

Plate 41

Stimulating Influence of the Virus on the Tar Cancers of Cottontail Rabbit W. R. 61 N (Table II).—

Figs. 15 to 20. The tumor on the ear was composed of three distinct types of carcinomatous tissue. In the hashed material used for implantation, some ordinary hair follicles were present. When the animal was killed 72 days later living cancer was found at only one of the four spots where the control implants had been put, and at this one as a degenerating layer on the wall of a minute cyst (Fig. 15; × 16). At the other three spots the growth had either wholly disappeared or died by keratinization (Fig. 16; × 12). The material exposed to virus on the other hand had given rise to large growths, three of them containing a great deal of cancerous tissue, with virus papilloma as well, while the fourth was composed wholly of the latter. The two kinds of growth were mostly separate, as in Fig. 17 (natural size). In the growth pictured the papilloma tissue consisted of well demarcated cysts filled with keratinized material, markedly striated and much of it melanotic, whereas the adjoining cancer was pale, irregularly lobulated, and dotted with necroses. From neither component could any virus be obtained. Another tumor was almost wholly cancerous (Fig. 18; × 12), and all three types of carcinoma present in the ear tumor were found in it (Figs. 19 and 20; × 74).

In this instance the virus greatly stimulated the cancerous cells but did not alter their morphology. The papillomatous tissue resulting from its action on the implanted fragments must have been derived from the hair follicle epithelium included in the cancerous tissue, since this latter contained no melanoblasts.



Photographed by Joseph B. Haulenbeek

(Rous and Kidd: Effects of papilloma virus upon implanted tar tumors)

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A TRANSPLANTABLE RABBIT CARCINOMA ORIGINATING IN A VIRUS-INDUCED PAPILLOMA AND CONTAINING THE VIRUS IN MASKED OR ALTERED FORM

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Plates 42 to 47

(Received for publication, April 2, 1940)

Carcinomas occasionally arise from the naturally occurring, cutaneous papillomas of cottontail rabbits (1), growths due to a virus discovered by Shope (2). This virus causes similar papillomas on inoculation into domestic, snowshoe, and jack rabbits (3), and in these foreign hosts its action results in malignancy far more often (4) than in cottontails. The cancers derive from the epidermal cells of the papillomas, whatever the host species (4), from elements that is to say which are proliferating as result of infection with the virus. These facts and others attest to the direct responsibility of the virus for the cancers, though whether it is their actuating principle is still uncertain.

Domestic rabbits would seem best suited to experiments on the relation of the virus to the malignant growths, since these are rare in cottontails, and jack and snowshoe rabbits do badly in captivity. But the virus strains which engender vigorous papillomas in domestic rabbits,—and it is only from such growths that cancer is likely to arise,—cannot ordinarily be recovered from the proliferating tissue (2).[1] None of our many attempts to procure virus from papillomas in which cancer had begun to appear at one spot or another has been successful, while results with the malignant growths themselves have also been negative. However, an antibody capable of neutralizing the virus *in vitro* appears sooner or later in the blood of nearly every animal carrying papillomas (5), and increases in amount as they enlarge. Its action upon the virus is specific, and it is absent from

[1] By testing many cottontail papillomas Shope procured some virus strains which could be passaged in domestic rabbits (*Proc. Soc. Exp. Biol. and Med.*, 1935, **32**, 830), but the papillomas produced on the successive inoculations appeared late, and enlarged slowly. The growths seldom became malignant and only after a long time, in our experience with them, and the virus was no longer recoverable from them when cancer appeared, nor could it be got from the cancers themselves (Kidd, J. G., and Rous, P., *J. Exp. Med.*, 1940, **71**, 469).

the blood of normal rabbits and those carrying tar tumors or the Brown-Pearce rabbit carcinoma (6). By serum tests one should be able to tell whether the virus—or perhaps an agent related to it antigenically,—exists and increases in the cancers.

A necessary first step in the undertaking would be to transplant the cancers, since their first hosts will have antibody in the blood as result of the preliminary virus papillomatosis. The transplantation of one cancer has already been reported (6) but the growth was lost on second transfer. The blood of two previously normal individuals in which it had grown was found to have a neutralizing effect on the virus, whereas that of controls was innocuous; but the neutralization was much less considerable than in the case of rabbits carrying papillomas. The present paper deals with another cancer which has been transplanted to fourteen successive groups of animals and now grows with great vigor. Like the previous tumor it has none of the morphological features of the virus-induced papilloma from which it came; yet the antibody which specifically neutralizes the virus appears in the blood of every individual in which the tumor progressively enlarges, and reaches a titer as great or greater than that attained in rabbits which have long carried large papillomas. These facts and their implications will be considered.

Material and Methods

The proliferation of virus papillomas is especially active in Dutch belted rabbits, and cancer follows with corresponding frequency (7). Hence rabbits of this variety were chosen for the work. They had been bought in open market for our previous efforts at transplantation, and only a single cancer grew after implantation in them out of six transferred to a total of 96 animals, and this one in but 2 of 17 individuals,—a finding not strange in view of the poor results with mouse tumors implanted in hosts of mixed stock, and the notorious difficulty of propagating squamous cell carcinomas. For present purposes a colony was developed from a single pair of Dutch "show" rabbits, and after many blood-related animals had become available some were inoculated with papilloma virus, and the cancers eventually arising from the growths thus produced were transferred to other individuals. To exclude any possibility that papilloma cells might be carried along together with the malignant elements, glandular metastases were utilized as material, and to increase the amount of it pieces of them were implanted in the leg muscles of the host. Growth in the muscles was usually rapid, nodules of cancerous tissue soon forming. These were cut fine in a mixture of Tyrode and host serum, additional Tyrode was added, and 1 cc. of the resulting suspension was injected into the upper extensor muscles of the forelegs and the anterior and posterior thigh muscles of new animals. The skin was slit prior to introduction of the injecting needle, to ensure that epidermal cells were not carried in on its point, perhaps to be infected with virus. Sometimes an injection was made also into one of the testicles, or a tumor fragment was placed in the anterior chamber of one eye, according to the technique used

by Greene (8). To ascertain whether active virus was present in the cancer which forms the subject of the present paper, most of the rabbits of the first ten tumor generations were inoculated with a 10 per cent Tyrode extract of part of the tumor hash, by rubbing the fluid into an area about 5 cm. square, freshly scarified with sandpaper, on the side of the body.

Several pairs of Dutch belted rabbits with typical markings and guaranteed to be pure bred were procured to start the colony. But the variations exhibited by their first offspring made clear that most were of mixed origin. Consequently all were discarded except a pair which had given litters with the characteristic markings described by Castle (9), and only those individuals were selected for the later matings which appeared typical. Their descendants had a pronounced heterogeneity nevertheless, some being spotted with gray or fawn, or having wall-eyes. Continued selection of individuals with typical markings has failed to exclude these variations, and the underlying genetic differences have been only too evident in the frequent failure of the transplantable cancer now under consideration to grow in some animals of a litter while it did so in others. Pieces of the metastases of ten primary cancers have been implanted in a total of 131 individuals, with only one success, and this was for a time precarious, as will be shown.

History of the Transplantations

The Primary Cancer.—The rabbit providing the tumor was a male, the offspring of F 5-82 and M 4, the progenitors of the colony. When it was 7½ months old, 10 per cent extracts of four different virus materials were inoculated into as many scarified spots, 4 by 6 cm. square, on each side of the body. Confluent papillomatosis developed at every situation and 9 months later one of the growths had begun to ulcerate and was extending deep. Three more underwent similar changes within the next month and a half, by which time the growth first mentioned had become a discoid, fleshy mass, 7.5 cm. across, ulcerated and foul. A firm nodule, 2 cm. in diameter, had recently appeared in the subcutaneous tissue about 4 cm. away from the nearest ulcerated tumor, in the direction of the axilla. It was excised and found to be encapsulated like a lymph node, and to consist of grayish-pink tissue with scattered, serpiginous necroses and a few small, well localized abscesses. A piece of the nodule was hashed and implanted in the forelegs and posterior thighs of the host. Thereafter the animal was twice transfused with large quantities of normal blood, but it was tottery on the 14th day after implantation and hence was killed. No other regional metastases existed, but several minute, glistening, gray nodules on the surface of the lungs proved microscopically to be secondaries. There was a fusiform nodule of malignant tissue, 2 cm. long, at one of the implantation sites, with smaller growths at the others.

Microscopic sections across the eight tumor masses on the sides of the rabbit showed that all had become malignant, and that in four the papilloma had been almost replaced by cancerous tissue. The axillary metastasis showed the same sort of cancer and so too did the implantation nodules in the legs.

First Tumor Generation.—The tissue of two of the largest leg nodules was hashed and implanted in the legs of 20 Dutch rabbits, and in an eye of 10 of them. All were of the first and second filial generations from F 5-82 and M 4, and siblings or cousins of the cancerous individual. During the next month a firm nodule 4 mm. across formed in the leg of rabbit 5-52 and three of the same size in the legs of 5-44; but they disappeared

within a few weeks. Rabbit 5-42 had at the end of the 5th week two spherical masses, 1.6 and 1.7 cm. across, in the forelegs and one of 0.7 cm. in a hind leg. They had not enlarged since the 4th week and for this reason the two largest were almost entirely removed for transplantation purposes. They grated slightly under the knife, projected beyond the muscle surface, and appeared to consist of finely striated tissue with scattered serpiginous necroses, surrounded by a thick zone of connective tissue, blurred at the margin. The microscope showed a carcinoma like the original but with numerous lymphocytes round about the epithelial islands and penetrating between their cells which in places appeared unhealthy. Everything indicated that retrogression of the tumors had begun, and the changes after operation substantiated this impression. The pieces left *in situ* rapidly disappeared and so too did the growth in the hind leg. The eye graft never grew and was gradually resorbed. The animal was subsequently used for breeding and it is still alive. The other 17 rabbits of the 1st Tumor Gen. remained negative.

Second Tumor Generation.—The tumors from D. R. 5-42 were hashed together and implanted in the legs of seven rabbits of the first and second filial generations from F 5-82 and M 4, and into F 5-82 herself as well as two non-related Dutch rabbits. All received also a tumor fragment in the anterior chamber of one eye, and the four males of the group were injected into one testicle. A single animal developed growths, namely, F 5-82 from which the colony had stemmed. She was by now 4 years and 3 months old. No tumors had appeared when she was examined 2 months after implantation, but when 2 more had elapsed a football-shaped mass, $6 \times 4 \times 4$ cm., was found in the muscle of the right, posterior thigh. Though fairly firm it seemed to fluctuate, and proved cystic on incision, much glairy, turbid fluid flowing forth from a large central cavity, followed by lumps of yellow, grumous material. The wall of the cyst was ragged, 1 to 3 mm. thick except toward the upper pole where was a solid boss projecting 5 mm. A slice was taken here for section. Microscopically it was found to consist almost wholly of connective tissue but with islands of carcinoma cells, showing many mitoses, along the wall of the cyst.

The cyst was left open but the skin was brought together with interrupted sutures. These became infected and the wound gaped yet healed almost completely in 2 weeks, no extension of the tumor into the subcutaneous tissue taking place. After another week the growth was larger than before, with a radish-shaped prolongation toward the hip. It was again cut into and the cyst was found to have reconstituted itself, once more containing glairy fluid and grumous, necrotic tissue, with a thicker wall. There was no sign at this time of bacterial infection. A large piece of the wall was cut away for transplantation and the skin brought together as before. Incidentally to the new transfers, rabbit 5-82 was injected with the suspension of tumor material it had itself provided, this time in the subcutaneous tissue of the groins and in the leg muscles at all five situations where the previous grafts had yielded no growths. A fragment of the tumor was also placed in the eye not previously utilized. The first intraocular graft had been almost completely resorbed.

Within the next month nodules appeared at four of the five intramuscular sites and the new intraocular graft had begun to grow. After a month and a half the original tumor mass was 6 cm. across, again a reconstituted cyst but with several solid nodules about it; and three of the new growths had much increased in size, the largest being 4 cm. across, spherical and fluctuating. The recently implanted anterior chamber of the

eye was half full of neoplastic tissue. During subsequent weeks the main tumor enlarged until it reached nearly from knee to hip, and by the 80th day after the second implantation the animal was thin and ill. It was transfused and the main tumor cut into, with the evacuation of much glairy fluid mixed with pus. Cultures showed a Gram-positive diphtheroid bacillus in the pus. The wound was left open to drain. The masses in the other legs continued to enlarge, reaching 6 cm. in diameter, though the growth in the eye looked less solid, as if resorbing. The animal weakened gradually despite a second transfusion, cachexia became marked, and death occurred 19 days after the last operation and 209 days after the first implantation. The axillary and iliac lymph nodes were devoid of metastases, but a large tumor embolus was found in one lung. It had lodged at an arterial fork, and the cells appeared in good condition and had almost penetrated the vessel wall. The growths in the legs were cystic and contained glairy fluid and opaque, yellow gouts. No tumors were found at the implantation sites in the groins.

Third Tumor Generation.—The material got at second operation from F 5-82 was injected into 28 of her descendants of the first, second, and third filial generations. She had been mated several times with M 4 and the offspring had been interbred or back-crossed. Six of the individuals now utilized had previously been implanted unsuccessfully with the original cancerous material, and in them the new implantations produced no growths. Progressively enlarging tumors were obtained, however, in six of the other 22 animals. Bits of the neoplastic tissue had been placed in an eye of twelve, with negative results, though four developed leg tumors while the intraocular grafts were disappearing.

Fourth Tumor Generation.—The 25 animals next employed were implanted at separate situations with tumor materials from two rabbits of the third generation, in order to spread the chances of success. These materials grew or failed in the same individuals.

Nineteen of the animals were of the first, second, and third generations from F 5-82 while the other six were of the gray brown (agouti) variety and had been bought at random. Two of the former and one of the latter developed tumors, thus providing opportunity for further transfers in both breeds. The cancer appeared at more situations in the agouti rabbit and enlarged faster.

Fifth Tumor Generation, Series A and B.—The growths provided by the blood-related, Dutch belted rabbits and the agouti individual just mentioned were transferred to 19 and 20 animals of these sorts respectively. Tumors developed in eight of the Dutch animals, with one retrogression subsequently, and in twelve of the agouti breed, with two retrogressions. Again the tumors appeared earlier and grew much faster in the agouti breed.

Sixth, Seventh, and Eighth Tumor Generations, Series A and B.—The cancer was by now well established in two breeds of rabbits, and it was transferred to three successive groups of each sort. It grew in more than half of the individuals implanted, appearing in 8 of the 10 agoutis of the 6th Gen. Animals of this sort (series A) regularly proved the more favorable, the cancer growing much faster in them though not in a greater number of individuals. Nine of the 10 animals comprising the 6th Gen. A were inoculated into a testicle, with negative results. For several of the transplantations the tumors of two individuals were employed, either together or separately. Those injected separately usually "took" in the same individuals, but the resulting growths often enlarged at differing rates.

Ninth to Thirteenth Generations.—Agouti animals have been relied upon entirely since the 8th Gen., and the tumor has continued to flourish. All of the 10 rabbits of the 9th Gen. and the 15 of the 11th Gen. developed growths, which retrogressed however after a few weeks in 6 and 2 individuals, respectively.

General Findings on Transplantation

The propagation of the cancer was uncertain in the beginning but it has gradually become sure. The growths providing material for the 2nd Tumor Gen. had begun to retrogress when used. In the next few groups of animals the cancer "took" in only an occasional individual of the large numbers to which it was transferred, and in favorable hosts it grew at only one or two of the six implantation sites. The differing character of the individual tumor fragments,—which often consisted almost wholly of reactive connective tissue,—doubtless had much to do with this. Later, as the proportion of cancer cells in the grafts increased, the results became less irregular, and of late tumors have regularly appeared wherever implantation was done in the leg muscles of favorable animals. Generally, though not always, the tumors in one individual behaved in the same way, growing or retrogressing together. A few animals were reimplanted with the tissue of their own enlarging tumors but in only one, the female progenitor of the colony (F 5-82), did new nodules result. Probably the host develops some secondary resistance to the cancer like that so often called forth by transplanted rat and mouse tumors. The tumor does badly in the subcutaneous connective tissue and in the anterior chamber of the eye,—where it has grown only once (again in F 5-82),—and a "take" has never occurred in the testicle.

The most rapidly enlarging cancers have regularly been chosen for transfer, and so swiftly have they grown of late as to cut down greatly the interval between transplantations. The tumor of the 1st Gen. was not utilized until the 139th day and those of the next few generations did not get big enough to provide material until 2 months or more had elapsed; but after the 5th Gen. large cancers were available within a few weeks, notably, in agouti animals, and the time between transfers has ranged between 17 and 40 days. The growth would not have killed until later but our aim was to pass the cancer through many successive hosts. Now growth is so rapid that frequently zeppelin-shaped nodules 2 cm. in diameter develop within 3 weeks. There is a critical period toward the end of this time when retrogression may occur,—the growths dwindling in one-third or more of the animals, so fast sometimes that in another 2 weeks they are gone. Retrogression is a rare event later, the cancers as a rule enlarging steadily. In the first tumor generations, when growth at single situations was frequent, the resulting cysts sometimes reached a diameter of 15 cm. or more and interfered with locomotion. Now, with tumors at all six implantation sites, death usually occurs by the time they are 8 to 10 cm. across. In one instance, of a half grown animal, the cancer killed in less than 6 weeks, and in an adult it did so in 2 months and a week. 10 to 15 weeks is the usual duration of life. Cachexia is very marked, owing doubtless to absorption from the large expanses of necrotic tissue on the walls of the cysts.

The original cancer rabbit had secondary growths in the lungs as well as the metastasis in a regional lymph node with which the transplantations were started; and F 5-82 of the 2nd Tumor Gen., an unusually favorable host, had a large embolus in a lung vessel, with cancer cells about to invade the alveolar tissue. No other animals showed

secondary growths until the 5th Gen. was reached, when nodules were found in the axillary and iliac glands of a few individuals. Since then metastases have been fairly frequent at these situations. In a Dutch belted rabbit of the 8th Gen. there were small pulmonary growths as well.

Many of the animals utilized for transplantation were newly weaned, Dutch belted rabbits, and some were half grown. They were selected because many transplantable tumors succeed best in young animals. Those we used were as nearly related to the original tumor animal as the adults implanted at the same time, yet the tumor grew in no greater proportion of them, though killing earlier.

It seems probable that the utilization of blood-related animals was responsible for the maintenance of the cancer in the first few groups of rabbits, but the results as a whole are like those ordinarily obtained when a tumor is propagated in animals of mixed genetic constitution, with selection for successive transfer of the growths that have done best. The gradual acquisition of the ability to proliferate in all hosts, even those of a different breed, the quickened rate of enlargement, and the gain in malignancy evidenced by earlier death and an increased frequency of metastasis formation, are all familiar phenomena to workers with transplanted growths.

In a recent experiment the neoplastic cells remained capable of forming growths after 24 hours at −22°C. though not at −70°. Some successful transplantations have been carried out by means of suspensions of the cancer cells, made by forcing the tissue through a fine sieve of monel metal and adding Tyrode solution.

Character of the Transplantable Tumor

As already mentioned, malignant changes occurred in all of the eight virus-induced papillomas of the animal from which the cancer came. It was impossible to tell from which tumor mass the metastasis had derived that was utilized as transplantation material, since all contained cancers of the same sort; but the likelihood narrowed to two of them. All were studied in cross-section; Fig. 1 illustrates the general findings. The progression there pictured from benign papilloma to squamous cell carcinoma by way of malignant papillomatosis has been the subject of several previous papers (7, 10, 4). It will be noted that the squamous cell carcinoma at the center of the tumor mass has penetrated deep, amidst much reactive connective tissue, and that small cysts are forming as result of necrosis of the cells prior to keratinization. The axillary metastasis utilized for transfer showed a cancer having the same traits (Fig. 2), and so too did the tiny pulmonary nodules though these latter were not yet cystic. In the leg tumors cysts were just beginning to form (Fig. 3). Mitoses were very numerous, and the growths were markedly desmoplastic.

The cancer has since retained its initial character (compare Figs. 3 and 4). As viewed in cross-section it consists of big cells which never keratinize, though they usually enlarge before they are overtaken by necrosis, and sometimes begin an abortive differentiation. The nuclei are also big, with much marginated chromatin, and the numerous mitoses are often pathological. The cells form expanses more or less widely separated by a profuse, new-formed connective tissue that obviously acts to limit their aggressive activities. When they are liberated from this, as when the tumor is cut up and transplanted, they often invade actively, extending in broad tongues between individual muscle fibers, and sometimes applying themselves directly to these and replacing their

substance, though never penetrating into them and along their interior as the most malignant of tumors do. Active invasion and destruction of this sort lasts no more than a few days, because a profuse reactive connective tissue proliferation soon walls off the tumor cells. While the wall is forming there may be edema outside it, as if from irritation (Fig. 4). The desmoplastic influence of the cancer is so great that however rapidly it burrows through the reactive tissue this still forms ahead, with result that the malignant cells seldom regain contact with normal structures, and then briefly and locally. On such occasions it may again extend between the muscle fibers and attack them. Ordinarily the layer of reactive tissue is 2 to 3 mm. thick and the cancer enlarges by continually invading it. In the absence of such aggression it becomes scar tissue, a tough, sharply defined capsule, and any tumor islands lying within it are compressed and usually die.

The extending carcinoma has a coarse pattern (Fig. 5): it grows out in blunt tongues which may broaden into large expanses of undifferentiated cells. There are scattered capillaries amidst these, each in a wisp of connective tissue (Fig. 6), and the neoplastic cells next the little vessels are often elongated and radial because of crowding. As proliferation continues those furthest away die and in consequence cysts form containing debris. The primary cancer had become cystic in this way, and so too had the glandular metastasis and the implantation nodules. When small the cysts may be forked or ramifying, according to the shape of the epithelial expanses in which they lie (Fig. 5). They enlarge progressively by death of the cells along their walls, and fluid soon begins to accumulate in them in addition to necrotic material. It gathers under considerable tension, as later palpation discloses, and hence the cysts become spherical and tend to coalesce with result in a large central cavity. If the cancer is very cellular the cavity may have a ragged lining. Until it reaches a diameter of several centimeters this lining may consist of living papillae (Fig. 7) having an origin diametrically different from that of the papillae which make up tar and virus papillomas. For they are the outcome of destructive not constructive activities,—mere remnants left by ischemic necrosis, their cores the blood vessels which had nourished the malignant cells, and their covering such of these latter as have survived by reason of a position near the blood stream. Occasional broader cores there are too, made up of the connective tissue which formerly lay between epithelial expanses now almost wholly dead. As the cysts enlarge further, the papillae become shorter, dying at the ends, and at length they are reduced to blunt protrusions by interior pressure (Fig. 8). Usually the cancer in the wall of the cyst continues to invade and break down, sometimes extending within lymphatics; and hence the cyst grows. In occasional instances the neoplastic epithelium becomes less and less able to penetrate the enveloping connective tissue and at length merely lines the cyst, and ultimately dies, with result that only encapsulated debris is left.

The intramuscular cancer of F 5-82 (2nd Tumor Gen.), as procured at first and second operation, had precisely the character of the implantation growths in the animal in which the tumor arose. It was a carcinoma with almost no tendency to differentiate, the cells dying early with result in cyst formation (Fig. 8). After the second operation, when the growth became infected with bacteria, its morphology changed. Its proliferating cells were no longer nearly alike in size and aspect but instead many were huge, multinucleate, or with giant nuclei (Fig. 17), while others were unusually small with no more than a rim of cytoplasm. Differences in tinctorial capacity were also great, some cells taking a deep color, others almost none. While these features were most marked

in the tumor subjected to operation they were present also at some spots in the other intramuscular growths, several of which contained pus as well as glairy and grumous material. There were fairly numerous polymorphonuclear leucocytes scattered amidst the reactive connective tissue. The intraocular tumor had a similar, unusual cytology, was partly dead, and obviously in process of resorption.

The changes in the cancer in this instance were doubtless due to the bacterial infection which became established in the tumor after the second operation; for similar pictures have been encountered now and again in tumors of the later generations in which bacteria were demonstrable. But the cellular abnormalities were not wholly confined to such growths; they have been found, affecting individual cells or small aggregates, even in those cancers which appear "healthiest."

In a considerable proportion of hosts, as already stated, the tumor retrogresses after proliferating rapidly for a few weeks. It ceases to invade, lymphocytes and macrophages accumulate about it, and some of the latter penetrate between its cells. Soon these begin to die and very swiftly all succumb, leaving islands of debris surrounded by foreign body giant cells amidst granulation tissue containing scattered polymorphonuclear leucocytes. Resorption of the dead material is rapid. The succession of histological events differs in no significant respect from that encountered with other transplantable tumors.

When proliferating in lymph glands the carcinoma exhibits its ordinary character, and becomes cystic on reaching a diameter of only a few millimeters (Figs. 9, 10). So long as its invasion is confined to the gland parenchyma no connective tissue reaction occurs, but this becomes pronounced once it has extended outside. The pulmonary metastases recently encountered have been minute (Fig. 11).

The way in which the tumor enlarges and breaks down brings singular consequences in the gross. When the cystic change is just beginning the neoplastic tissue appears solid on section, firm, close-textured, with scattered, yellow, serpiginous necroses and dots (Fig. 12). But by the time the growth has reached a diameter of 3 to 4 cm. a central cyst forms (Fig. 13), soon comprising much of its bulk (Fig. 14), and this cyst is a dominating feature thereafter (Figs. 15, 16). While still solid, the tumor has a fusiform, football, or radish shape, with its long axis in the direction of the muscle fibers, but later it becomes approximately spherical. If several nodules have arisen at one implantation site, as occasionally happens, each becomes cystic and they and the cysts they contain may coalesce if the animal lives long enough. At most situations though, a solitary cyst forms which in due course becomes large. Its wall of cancerous tissue is from 2 to 10 mm. thick, so firm sometimes as to grate under the knife, and variegated with serpiginous necroses or small cavities containing soft, necrotic material. The lining of the cyst appears ragged and partly necrotic at first, for reasons already given, but later it is studded with what look like giant pink granulations, these being actually covered with a thick layer of proliferating carcinoma cells, as the microscope shows. The contents of the cyst is peculiar, a clear, glairy fluid like thinned white of egg, with necrotic, yellow masses lying amidst it. It accumulates under a pressure which keeps the cyst tense. Sometimes the fluid is thickened and rendered turbid by finely dispersed, dead cells, and it may then have the ground-glass aspect of solidifying candle grease; or it may be light brown, or pink or chocolate-colored with pigment from the blood of papillae that have undergone necrosis. Usually though, the fluid is pellucid and colorless. The necrotic material has ordinarily the form of gouts or yellow, cus-

tardy lumps but occasionally it is clayey or pultaceous. The pressure within the cysts is so great that when the tumor extends through the muscle aponeurosis, as occasionally happens, herniation promptly follows and a dissecting cyst forms in the subcutaneous tissue. This is always flaccid and its wall is thin at first; for as yet there has been no extension outwards of the tumor but only a gushing forth of dead material. Later the cyst may acquire a carcinomatous lining. As a rule the cancer does badly in the subcutaneous tissue, seldom extending into this after incisions to procure material; and the skin has never been invaded despite the numerous opportunities that biopsies provided. On the one occasion when the cancer established itself after implantation in the anterior chamber of the eye, it eventually retrogressed. In this connection it may be recalled that two highly malignant cottontail cancers of a previous paper (4) also failed to grow after transfer to the anterior chamber.

In a few animals of recent tumor generations the cancer has assumed a new aspect, though only locally. At some spots it no longer grows in thick, blunt tongues but its invading cells extend through the connective tissue in narrow strands and die before multiplying into masses (Fig. 18). The ultimate in anaplasia would seem to have been achieved in this change. It was first noted in a tumor of the third generation which had grown for many months, and it has now become frequent. Sometimes it is obviously determined by inflammation of the supporting tissue, but in most cases it exists side by side with cancer of the ordinary type, apparently under the same conditions, as if it were the outcome of a discontinuous alteration in the neoplastic cells. Efforts to procure completely anaplastic tumors by selective transplantation are under way.

The first good-sized cancer obtained by transplantation (rabbit F 5-82 of the 2nd Gen.) would have been mistaken for an old abscess had not this error been made with the carcinoma previously propagated (6), which likewise gave rise to a cystic growth containing glairy fluid mixed with yellow, necrotic debris. This material and the profuse, reactive connective tissue were suggestive of bacterial infection, but none was demonstrable by cultures or stains. Our repeated attempts to procure bacteria from the tumor now under consideration have had no better success, save in a few cases in which there was pus in the cyst in addition to the ordinary contents. A Gram-positive diphtheroid was found in one of these, and unidentified Gram-negative rods in two others. The search for bacteria has involved taking cultures on a variety of media, including broth and agar to which rabbit's serum had been added, and repeated examination of the sediment of tumor extracts, as obtained by centrifugation and colored with methylene blue, Gram, or the Giemsa stain.

Tests for the Presence of Virus

The four virus materials inoculated into the rabbit providing the tumor for transplantation had been employed in previous experiments and were known to cause vigorous papillomas from which no virus was recoverable. Nor has it been got from the transplanted cancer. Every animal of the first ten tumor generations, except those of the 7th Gen. series B and the 9th Gen., was inoculated into the scarified skin with a 10 per cent extract of a part of the hashed tissue utilized for implantation. In none did skin growths arise.

In a further effort at direct demonstration of the virus a 5 per cent Tyrode extract was made of a cancer of the 11th Tumor Gen. and inoculated into the ears of nine rab-

bits, after it had been centrifuged to remove gross particles. The ears had been tarred over the inner surface twice weekly for 17 weeks and tar papillomas had appeared there in greater or less number. The organs were thus prepared because experiment had shown that when active papilloma virus is injected into animals thus treated it localizes abundantly in the tarred epidermis, causing some of the benign papillomas already present there to become cancers, and engendering both benign and malignant tumors where none were previously visible (11). In the present instance it seemed possible that a "masked" virus might be extracted from the cancerous tissue which would prove capable of infecting cells prepared by tarring, though powerless with normal elements. To ensure that the presumptive virus reached the epidermis, one ear of each animal was infiltrated with 10 cc. of the extract by way of a marginal vein, after the circulation had been shut off at the base with a rubber band, and the other ear was infiltrated under like circumstances with the same amount of Tyrode solution. Both organs were tarred once immediately after. When active papilloma virus is introduced under the conditions given innumerable growths soon appear (12), but in the experiment now described none arose that could be attributed to the injected material.

Several indirect methods were available to test for the presence of the papilloma virus in the cancers,—inoculation of the cancer animals with it to learn whether they had acquired any resistance, examination of their blood for the presence of specific antiviral antibody by means of the neutralization (5) and the complement fixation (13) tests respectively. Previous experiment had shown inoculation to be the most delicate of the three methods, disclosing very slight amounts of resistance; but use of it as routine would have defeated our aim to remove the cancer far from possible contamination with the virus. The neutralization test comes next in sensitiveness (14) and hence was largely relied upon. It gave clear cut results (Figs. 19, 20).

The *neutralization test* has been used in much previous work (5, 4). The serum of each rabbit examined in the present relation was mixed with an equal amount of a Berkefeld filtrate of a 5 per cent Tyrode extract of glycerinated, cottontail papilloma tissue; and after 2 hours at 37°C. the mixtures were rubbed into freshly scarified squares on the belly or side of each of three domestic rabbits. A control mixture with Tyrode instead of serum was also inoculated. The extracts were all capable of promptly producing confluent papillomatosis as the control tests proved. They were made from the papillomas of several cottontails in order to broaden the conditions. The squares, checkering the belly and sides of the rabbits, were dried in a blast of warm air and covered individually with sterilized gauze until healing had occurred, to exclude any possibility of transfer of the inocula. All specimens that were to be directly compared were rubbed into the same animals. After healing had taken place the squares were examined every 3 to 5 days, until no more growths appeared, that is to say, until the 36th to 45th days.

The neutralizing power of the sera tested showed itself not only in an absence of papillomas, or reduction in their number on the inoculated areas, but also in a delay in their appearance. Two records of the findings are presented in each of the tables (Tables I to VII), one obtained early, while the growths were appearing, and the final reading of their total number. As controls to each test of the tumor animals of Dutch belted stock, serum specimens were included which had come from rabbits of the same implantation which had failed to develop growths, as also specimens from normal rabbits which were their sibs or cousins. In the later experiments with agouti animals only normal controls

were available because the implantations regularly resulted in growths. To learn how the resistance of the cancer animals compared with that induced by virus papillomas, serum specimens from individuals carrying such growths were included in several of the experiments.

The complement fixation test involves the same antigen as the neutralization test, namely the virus (14), but it does not disclose the presence of antibody in quite as small amount. It gives immediate, quantitative results though, and hence was employed as

TABLE I

Neutralization Tests with Serum of Rabbits Bearing Transplanted Cancers

(First Tumor Generation)

Source of serum	Rabbit No.	Number of tumors carried	Diameters of tumors	Growths from mixtures of serum and virus W. R. 54 in rabbits A, B, C					
				21st day			45th day		
				A	B	C	A	B	C
Rabbits			*cm.*						
With cancers (implanted 39 days)	5-42	Three	1.7 - 1.6 - 0.7 (T)	0	0	±	0	0	±
	5-44	Three	0.4 - 0.4 - 0.4*	0	0	0	++±	+	±
	5-52	One	0.4*	+++	+++	++	+++	+++	+++
Implanted but negative	5-38			+++	+++	++	++++	+++	+++
	5-46			+++	+++	++	++++	+++	++++
	5-50			+++	+++	++	++++	+++	++++
Normal rabbits of same stock	5-88			+++	+++	++	++++	+++	++++
	5-86			++±	++	++	++++	+++	++++
	F 5-82			+++	+++	++	++++	++±	++++
Tyrode control..				+++	+++	++±	++++	++±	++++

(T) = the tumors of D. R. 5-42 were used for transplantation on the 40th day.

++++ = confluent papillomatosis.

+++ = semiconfluent papillomatosis.

++ = many discrete papillomas.

+ = 5 to 10 " "

± = 2 to 4 " "

± = solitary discrete papilloma.

* Retrogressed later.

routine with all of the animals except those of the first two generations, both as a check upon the neutralization tests and whenever the latter seemed unnecessary. The technique has already been described and the reliability of the method demonstrated. The results obtained with it in the work now under discussion were essentially corroboratory to the neutralization test, and hence they will not be tabulated save in the case of the animals of the 5th and 6th Gen. B, for which no neutralization data are available.

Table I gives the outcome of the neutralization tests with the animals of the 1st Tumor Gen. It will be seen that the serum of rabbit 5-42, which carried large tumors, did away almost completely with the pathogenicity of the virus mixed with it, that the specimen from rabbit 5-44 with three very small growths had less effect, while that from

rabbit 5-52 with one such growth had none at all. The sera of the normal controls and of the implanted animals which failed to develop tumors were likewise devoid of effect (Fig. 20).

Only one animal of the ten inoculated for the 2nd Tumor Gen. developed a growth; this was rabbit F 5-82 which figured in the comparison of Table I. Immediately after it was bled as a control in this comparison it was implanted with the cancer of rabbit 5-42. A single growth resulted and from time to time as this enlarged tests were made of the host's blood. Table II is concerned entirely with the changes they disclosed. The serum specimen procured on the 118th day, when the tumor was 5 cm. across, was kept in the cold until the 139th day and tested with serum taken then; for preservation during many weeks does not diminish the antibody titer, as tests directed to the point have shown. Both sera were now found to have moderate neutralizing power in contrast to the ineffectiveness of the previous specimen. Control sera from two normal, blood-related rabbits and two implanted with the same material as 5-82 but with negative results, proved devoid of effect on the virus. These findings have not been included in the table.

On the 140th day rabbit F 5-82 was reimplanted with its own growth, with result in four new nodules 14 days later. The primary growth was still below its size prior to the excision of material. The blood now had a pronounced neutralizing power (154th day), and so too on the 202nd day. One of the new nodules had then disappeared but the others had enlarged further. In these later tests, three and five normal animals were included, respectively, as well as two on each occasion that had been implanted when F 5-82 first was, with negative results. None of the sera of these controls had the slightest neutralizing influence, and they have been omitted from the table.

In the 3rd Tumor Gen., six animals developed growths, but one died before the 63rd day when blood tests were carried out (Table III). The three that had several large tumors all yielded sera with marked neutralizing capacity, as did rabbit 6-92 in which only one growth was present and this only 6 mm. across. The blood of rabbit 6-91, on the other hand, contained no demonstrable antibody although it had a growth 1.8 cm. in diameter; but the antibody appeared later, as Table IV will show. The sera from the controls proved wholly devoid of effect. Among them were two animals (5-36, 5-48) which had been implanted for the 1st Tumor Gen. with negative results. (They had not been amongst those from which serum was taken for the test recorded in Table I.) To extend the comparison sera from five agouti rabbits, each with several papillomas amounting together to a large mass, were introduced into the experiment. The neutralizing capacity of their bloods proved to be no greater than that of the animals with big cancers.

Table IV summarizes the comparison carried out with animals of the 4th Tumor Gen. Incidentally the serum of rabbit 6-91, of the 3rd Gen., was again tested. Its cancer was now, on the 165th day, 3 cm. across, and its blood, previously without neutralizing effect, exerted this pronouncedly. The other results of the table corroborate and extend the previous findings. Cancer rabbit 7-90 was an agouti, the first of its kind in which the tumor had been propagated. The others, including the animals with papillomas, were from the Dutch belted colony. It will be seen that four of the five cancer animals of the 4th Gen. had blood with definite neutralizing capacity, but that this was lacking in the case of the fifth (7-73) which had had for a brief time a single small nodule which disappeared about 2 weeks before the test was made. One of the

papilloma animals (5-48), with four large growths when bled, yielded serum of only slight antiviral power.

TABLE II

Neutralization Tests with Serum of a Rabbit with Enlarging Cancers

(Second Tumor Generation—Rabbit F 5-82)

Time of test	Tumor size	Growths from mixtures of serum and virus in rabbits A to L					
		21st day			45th day		
		A	B	C	A	B	C
	mm.						
Prior to implantation		+++	+++	++	++++	++±	++++
Tyrode control		+++	+++	++±	++++	++±	++++
		21st day			42nd day		
		D	E	F	D	E	F
Implanted 118 days	50	+−	+	±	+	++	+
139 days	50 (T)	+	+	±	++	+++	+
Tyrode control		++±	+++	+++	++++	++++	++++
		G	H	I	G	H	I
Implanted 154 days	30						
Reimplanted 14 days	4 6* 6 10	0	±	±	+	±	0
Tyrode control		++±	+±	++±	++++	+++	+++
		J	K	L	J	K	L
Implanted 202 days	61						
Reimplanted 62 days	15 27 50	0	±	0	±	±	0
Tyrode control		+++	+++	++++	++++	++++	++++

(T) = the tumor of rabbit F 5-82 was used for transplantation on the 139th day.

Virus procured from the papillomas of W. R. 54 was used in the test prior to implantation. In the second test virus W. R. 56 + 1-56 was used, and in the third and fourth tests virus W. R. 1-10.

* Retrogressed later.

In the 5th Tumor Gen. A "takes" and progressive growth were obtained in ten agouti animals. Blood from all of these was compared with that from three of the nine nega-

TABLE III

Neutralization Tests with Serum of Rabbits Bearing Transplanted Cancers

(Third Tumor Generation)

Source of serum	Rabbit No	Tumor size	Growths from mixtures of serum and virus W. R. 1-10 in rabbits M, N, O					
			21st day			42nd day		
			M	N	O	M	N	O
		mm.						
Rabbits with cancers (implanted 63 days)	6-78	13 28 28 30 35	±	0	0	±	0	0
	6-82	15 (T) 16 25 28	0	+	0	±	+	0
	6-69	11 (T) 12 12 18 20	±	+	0	±	±	±
	6-91	18	+++	+++±	+++	++++	++++	++++
	6-92	6	0	±	0	±	±	+
Implanted but negative	5-36		++±	+++±	++	++++	++++	++++
	5-48		++±	++	++±	++++	++++	++++
	6-81		++±	++±	+++	++++	++++	++++
	6-85		++	+++	++	++++	++++	++++
	6-87		+++	+++	++±	++++	++++	++++
Normal rabbits of same stock	7-70		+++	+++	++±	++++	++++	+++±
	7-71		++±	+++	++±	++++	++++	++++
	7-72		++±	+++	+++	++++	++++	++++
	7-73		++	++++	+++	++++	++++	++++
	7-74		+++	+++	+++	++++	++++	++++
With virus-induced papillomas (inoculated 84 days)	F 1 F 2 F 6	8 large growths on each (3-4 cm. across)	± 0 0	± 0 0	0 0 0	± 0 ±	± 0 ±	0 0 ±
Inoculated 190 days	6-13	4 large growths	0	0	0	0	0	0
Inoculated 48 days	7-07	16 small growths	0	±	0	0	±	0
Tyrode control........................			+++	+++	++++	++++	++++	++++

(T) = the tumors of rabbits 6-82 and 6-69 were used for transplantation on the 65th day.

TABLE IV

Neutralization Tests with Serum of Rabbits Bearing Transplanted Cancers (Third and Fourth Tumor Generations)

Source of serum	Rabbit No.	Tumor size	Growths from mixtures of serum and virus W. R. 1-28 in rabbits A, B, C					
			18th day			43rd day		
			A	B	C	A	B	C
		mm.						
Rabbits with cancers of 3rd Gen. (implanted 165 days)	6-91*	30	0	0	0	±	±	0
Rabbits with cancers of 4th Gen. (implanted 99 days)	7-72	35 (T) 30 13 6 2	0	0	0	±	0	0
	7-70	7**	0	0	+	+	+	+
	7-89	36 (T)	±	0	0	±	++	+
	7-90[a]	23 (T)	+	++	±	++	+++	++±
	7-73	14*	+±	+++	++++	+++	++++	++++
Rabbits implanted for 4th Gen. but negative	7-71		++++	+++±	++++	++++	++++	++++
	7-74		++++	+++	++++	++++	++++	++++
	7-83		++++	+++	+++	++++	++++	++++
	7-87		++++	+++	++++	++++	++++	++++
Normal rabbits of same stock	5-52		++++	+++	++++	++++	++++	++++
	5-37		++++	+++	++++	++++	++++	++++
	5-35		+++±	+++	++++	++++	++++	++++
Rabbits carrying 4 virus-induced papillomas 3 to 8 cm. across (inoculated 94 days)	5-85		0	0	0	±	±	0
	5-46		±	0	±	+	++	+
	5-50		±	0	+	+±	++±	++±
	5-48		+±	0	+++	++±	+++	++++
Tyrode control			++++	+++	++++	++++	++++	++++

[a] Agouti rabbit.

(T) = the tumors of rabbit 7-90 were transplanted on the 61st day (series A), those of 7-72 and 7-89 on the 69th day (series B).

* Failed to manifest antibody in previous test (Table III) when the tumor was smaller.

** Disappeared before 87th day.

tive agouti rabbits of the same implanted group, as also with specimens from three normal individuals, and from five agouti rabbits carrying several papillomas each (Table V). Of the three rabbits inoculated with the test mixtures, one animal, C, was killed on the

27th day to obtain a picture illustrating how decisive the findings were (Fig. 19). The blood of all of the cancer rabbits had definite neutralizing power, though somewhat less marked in two instances than that of the rabbits with papillomas. All except one of the latter had carried its growths for many months whereas the cancers had been present only 60 days.

TABLE V

Neutralization Tests with Serum of Rabbits Bearing Transplanted Cancers

(Fifth Tumor Generation Series A,—Agouti Animals)

Source of serum	Rabbit No.	Duration of growths	Diameter of tumors	Growths from mixtures of serum and virus W. R. 1-56 in rabbits A, B, C							
				17th day			26th day			36th day	
				A	B	C	A	B	C*	A	B
		days	*cm.*								
Rabbits with cancers	8-46	60	4 - 4 - 2.5 - 4 - 3 - 4	0	0	0	0	0	0	±	±
	8-54	"	3 - 3.5 - 2 - 3.5 - 3	0	0	0	0	0	0	0	±
	8-50	"	5 - 4 - 4 - 1.5 - 4 - 4	0	0	0	±	±	0	±	±
	8-49	"	5 - 2 - 2 - 3 - 2.5	0	0	0	±	±	0	±	±
	8-55	"	2.5 - 2.5 - 2.5 - 1.1 - 1.5 - 1.4	0	0	0	±	0	±	±	0
	8-60	"	1.2 - 1 - 1.8 - 1 - 1.6 (T)	0	0	0	±	0	±	±	±
	8-62	"	4 - 5 - 4 - 4 - 3 - 5 (T)	0	0	0	±	+	0	0	+
	8-61	"	3 - 3.5 - 3.5 - 3 - 4	0	0	0	±	+	±	±	+
	8-59	"	3.5 - 5 - 3 - 5 - 4 - 6	0	0	0	+	+±	+±	+	++
	8-45	"	3 - 4 - 3 - 4 - 4	0	0	0	++	+	+±	++±	+
Rabbits implanted with same material but negative	8-44			++	+++	+++	+++	++++	++++	++++	++++
	8-51			++	+++	++	+++	++++	++++	++++	++++
	8-56			++	+++	++	+++±	++++	+++±	++++	++++
Normal animals of same breed	9-07			+	+++	+++	+++	++++	+++±	++++	++++
	9-08			+±	+++	+++	+++	++++	++++	++++	++++
	9-09			++	+++	+++	+++±	++++	++++	++++	++++
Rabbits with virus-induced papillomas	1-39	51	9 growths averaging 3 cm.	0	0	0	+	±	±	+	±
	6-12	315	6 - 6 - 6 - 8	0	0	0	±	0	0	±	0
	6-16	"	3.5 - 3.5 - 3.5 - 3.5	0	0	0	±	0	0	±	0
	6-10	"	6 - 6 - 6 - 6	0	0	0	+	±	±	+	+
	4-88	396	2 - 2 - 2 - 3	0	0	0	±	0	0	±	±
Tyrode control				++	+±	++±	+++	+++±	+++±	+++±	+++±

(T) = the tumors of rabbits 8-60 and 8-62 were used for transplantation on the 23rd day.

* Killed on 27th day for photographic purposes (Fig. 19).

Every rabbit thus far in which the cancer grew large had yielded serum with marked capacity to neutralize the virus. In the absence of any sign of change in this respect, the decision was made to do no more neutralizations until after the tumor had undergone several further transfers. Complement fixation tests were performed, however, with the sera of the cancer rabbits of the 5th and 6th Gen. B (Dutch belted animals). For comparison specimens were taken from two individuals of the 5th Gen. and three of the 6th Gen. which had not developed growths. At the same time eleven Dutch belted rabbits carrying virus-induced papillomas were tested. Some had served previously

TABLE VI

Complement Fixation Tests with Serum of Rabbits Bearing Transplanted Cancers or Virus-Induced Papillomas

(Fifth and Sixth Generations, Series B,—Dutch Belted Rabbits)

Source of serum	Rabbit No.	Time since implantation or inoculation	Size of growths	Complement fixation titer of serum				
				1:4	1:8	1:16	1:32	1:64
		days	*cm.*					
Rabbits with cancers (5th Gen.)	8-65	95	10 — 8	++++	++++	++++	++++	++++
	8-66	"	3.5 — 12 — 10	++++	++++	++++	++++	++++
	8-72	"	6 — 6 — 8 — 6 — 6 — 10	+++±	++++	++++	++++	++++
	8-79	"	8 — 4 — 10 — 7 — 3 — 4.5	++++	++++	++++	++++	++++
	8-74	74*	5 — 9 — 5 — 7 — 5 — 6 (T)	++++	++++	++++	++++	++++
	8-78	76*	5 — 4 — 6 — 5 — 4.5 — 4	+++±	++±	0	0	0
Implanted but negative	8-63	95		0	0	0	0	0
	8-64	"		0	0	0	0	0
Rabbits implanted with cancer (6th Gen.)	9-17	62	3.5 — 2.5 — 5 — 3 — 4	++++	++++	++++	+	0
	9-19	41*	2.5 — 2.5 — 5 — 2.5 — 2.3 — 3	++++	++++	++++	+++±	0
	9-20	62	3.5 — 5 — 2.5 — 8 — 5 — 5	++++	++++	++++	++++	++++
	9-22	"	4 — 4.5 — 8 — 3.5 — 5.5 — 3	++++	++++	++++	++++	++++
	9-24	"	4.5 — 4.5 — 7 — 4 — 3	++++	++++	++	0	0
	9-21	"	3 — 5	++++	++++	++++	++++	+++±
	9-26	"	2.5 — 3.5 — 3.5 — 4.5 — 3.5 — 3.5	+++±	++++	++++	++++	++++
Implanted but negative	9-18	"		0	0	0	0	0
	9-23	"		0	0	0	0	0
	9-25	"		0	0	0	0	0
Rabbits with virus-induced papillomas	5-45	154	5 — 7 — 6 — 5	++++	++++	++++	+++±	0
	5-46	"	6 — 7 — 6 — 7	+++	0	0	0	0
	5-51	"	6 — 7 — 6 — 5	++++	++++	+	0	0
	5-80	"	6 — 7 — 8 — 6	++++	++++	++++	+	0
	5-85	"	4 — 7 — 6 — 5	++++	++++	++++	+++±	0
	5-86	"	8 — 8 — 8 — 8	0	0	0	0	0
	5-88	"	8 — 8 — 8 — 7	+++±	++++	++++	+++	±
	6-75	121	6 — 8 — 8 — 7	++++	++++	++++	0	0
	6-81	"	6 — 6 — 8 — 6	++++	++++	+	0	0
	6-83	"	7 — 8 — 6 — 8	++++	++++	+	0	0
	6-89	"	12 — 10 — 12 — 10	++++	++++	++±	0	0

(T) = the tumors of 8-74 were used for transplantation on the 44th day.
++++ = complete fixation of complement (no hemolysis).
0 = no fixation of complement (no hemolysis).
2 units of complement in all tubes.
None of the sera was anticomplementary in control tests, nor was the antigen.
Antigen, W. R. 1-30 E, Berkefeld filtrate, 1:120.
* Moribund when bled on this day.

as normal controls while others had been implanted with the cancer as part of the groups of the 1st, 2nd, or 3rd Gen., but had failed to develop growths. The blood of a few had been tested previously and had proved devoid of the neutralizing property (*vide* rabbits 5-46, 5-86, Table I, and rabbit 6-81, Table III); and later inoculation with the virus had produced vigorous papillomas in them all.

The serum of every cancerous animal was found to fix complement strongly, whereas the results were negative with the specimens from the implanted controls (Table VI). Fixation was obtained with the serum of all except one of the papilloma rabbits, but it was wholly lacking in the case of this one, while in another it was slight, and the titer

TABLE VII

Neutralization Tests with Serum of Rabbits Bearing Transplanted Cancers

(Seventh, Eighth, Ninth, and Tenth Tumor Generations)

Source of serum	Rabbit No.	Time since implantation with cancer or inoculation with virus	Number of growths carried	Diameter of growths	Growths from mixtures of serum and virus W. R. 1-10 (Test rabbits A, B, C)					
					19th day			42nd day		
					A	B	C	A	B	C
		days		*cm.*						
Rabbits										
with 7th generation cancers (series B)	10-16	59	Five	5.0 - 4.5 - 5.0 - 5.0 - 4.0	0	0	0	0	0	±
	10-17	"	Three	2.5 - 3.5 - 0.8	0	0	0	0	0	0
	10-18	"	Four	5.0 - 5.0 - 6.0 - 3.0	0	0	0	±	±	0
	10-19	"	Six	4.0 - 4.0 - 6.0 - 5.0 - 4.0 - 3.0	0	0	0	0	0	0
with 8th generation cancers	9-65	86	Six	3.8 - 7.0 - 9.0 - 8.0 - 6.0 - 6.0 (T)	0	0	0	0	±	±
	9-67	"	Two	3.5 - 5.0	0	0	0	±	0	0
with 9th generation cancers	10-27	59	Six	5.0 - 5.0 - 6.0 - 6.0 - 4.0 - 4.0	0	0	0	0	0	0
	10-28	"	Five	6.0 - 7.0 - 3.5 - 6.0 - 2.8	0	0	0	±	0	0
with 10th generation cancers	10-37	47	Three	2.0 - 1.5 - 1.5	0	+	0	±	+	0
	10-38	"	Six	3.5 - 4.0 - 4.0 - 2.8 - 3.5 - 3.5	0	0	0	0	±	±
	10-40	"	Six	3.0 - 3.5 - 3.5 - 3.5 - 3.0 - 4.0 (T)	0	0	0	±	0	0
with virus-induced papillomas	10-07	67	Five	3.5 (average)	0	0	0	0	+	±
	10-08	"	Six	4.0 "	0	0	0	0	0	0
	10-09	"	Five	3.5 "	+	+	0	++	+++	0
normal	10-61				++±	++±	±	++++	++++	±
	10-62				++	+++	+	++	++++	+
	10-63				+++	+++±	±	++++	++++	±
Tyrode control					+++	++	0	++++	++++	±

(T) = the tumors of rabbit 9-65 were used for transplantation on the 27th day; those of rabbit 10-40 on the 91st day.

for the group as a whole was less than in the case of the cancer rabbits although these had had their growths for a shorter time.[2]

[2] To avoid confusion it should be pointed out that the plus signs of Table VI record the effectiveness of the blood antibody, as indicated by its capacity to fix complement in mixture with the virus, whereas in the neutralization tables these symbolize its ineffectiveness,—representing as they do there the fact that the mixtures of blood and virus gave rise to growths in greater or less number.

The neutralization tests were resumed after the cancers in the animals of the 10th Gen. had grown big. One of the group had been killed for transplantation purposes, leaving three with tumors. These were tested together with three normal agouti rabbits, three that carried papillomas, and all of the still surviving cancerous animals of *the 7th Gen. B (Dutch belted rabbits) and of the 8th Gen. A and 9th Gen. A (agouti* animals like those of the 10th Gen.). Table VII gives the results. It shows that the sera of the cancerous individuals,—each of which had good-sized tumors,—neutralized the virus markedly in every instance. The serum of one of the papilloma rabbits was relatively ineffective by comparison. One of the animals inoculated with the test mixtures (rabbit C) proved highly exceptional in possessing some natural resistance to the virus; it developed but few growths in response to inocula that caused many on rabbits A and B.

In brief the blood of every animal in which the cancer reached a considerable size had a definite and usually a marked power to neutralize the papilloma virus. Blood from normal animals and from those in which the tumor failed to grow was, on the other hand, devoid of effect under the conditions of our tests. When only small tumors had appeared the virus-neutralizing antibody was sometimes absent, but it developed later in the blood of those animals in which such tumors enlarged (Tables II and IV). The antibody titer attained seemed on the whole to be slightly higher than that reached in animals which had carried large virus-induced papillomas for a much longer time. There was no falling off in this titer as the propagation of the tumor went on. It became as high in the animals of the 10th Tumor Gen., tested a year and 4 months after the initial transfer of the tumor, as in the first new host in which it grew.

DISCUSSION

The tumor here considered is a squamous cell carcinoma, both in morphology and in all the phenomena of its enlargement and metastasis. Furthermore the cellular reaction occurring when it retrogresses is like that taking place about other transplantable neoplasms under similar circumstances. In previous papers the fact has been stressed that the cancers deriving from virus-induced rabbit papillomas tend in general to alter from one form to another in the direction of greater anaplasia (7, 4), and that in proportion as they do this the papillomatous traits usually disappear. They were gone from our cancer before it was first transferred, but it had not then wholly lost the ability to differentiate, and only of late and inconstantly has it become completely anaplastic.

The cancer forms large, solitary cysts containing quantities of glairy fluid under tension, in addition to necrotic debris. Another transplanted cancer deriving from a virus

papilloma did the same (6), and cysts with glairy contents have been encountered in two out of twenty cottontail cancers of similar origin (4),—from all of which one may infer this feature to be characteristic of certain tumors of rabbits. Only three epidermoid carcinomas have been successfully transplanted as yet in these animals, the present cancer, the one just referred to, and the Brown-Pearce carcinoma. This last seems to have arisen from a hair follicle cell or cells (15), and histologically it differs distinctively from our two tumors. It is rarely cystic, never contains glairy fluid, and the papilloma virus fails to persist after introduction into it (6).

The identical character of the two tumors we have transferred leads one to ask whether all cancers arising from the virus papillomas of domestic rabbits will not manifest similar traits on propagation. In favor of this possibility is the "pure line" origin of the growths from epidermal cells altered in a distinctive way by the papilloma virus (7, 16), as further their tendency to undergo changes later in a single direction, with anaplastic squamous cell carcinomatosis as the end product. But the cancers exhibit a considerable diversity within the limits outlined (7, 10, 4). A number of them have been successfully transplanted to the leg muscles of the animals in which they arose, with result in large growths in some cases (7, 10), and none of these growths thus far has contained cysts full of glairy fluid. The desmoplastic influence of the cancer now under propagation, while a striking feature of its growth, is no greater than that of a considerable proportion of human epidermoid cancers or of many of the cancers that we have elicited by tarring the skin of cottontail rabbits.

The ability shown by the tumor to flourish in hosts of a different breed after it had been propagated for a time, is no new finding with growths rendered increasingly effective by selective transplantation; but that it did better in alien animals than in blood relations of the original host is a highly exceptional phenomenon, though not without precedent. A spindle cell sarcoma of the fowl, rifted with blood sinuses and with a tendency to metastasize to the muscles, has been found to attain far greater success on transplantation to birds of the Plymouth Rock variety than in brown leghorns like the original host (17). The sarcoma proved due to a virus, and the possibility cannot be ruled out that it enlarged by an unobserved, secondary infection of neighboring elements as well as by intrinsic cell proliferation, in which case its remarkable success in Plymouth Rocks might have been consequent in part on a greater susceptibility of these birds to the virus. No such explanation will hold for the present rabbit carcinoma which can only have enlarged in the leg muscles by multiplication of the epithelial elements introduced there. Nor will the explanation hold for a tumor of Japanese mice which failed to grow in ordinary white mice but succeeded in hybrids (18).

As the transplanted cancer enlarged, an antibody capable of neutralizing the papilloma virus *in vitro*, and of fixing complement in mixture with the virus, regularly appeared in the blood of the new hosts, just as happens in animals with enlarging virus papillomas (5). This antibody was not present in normal rabbits or those in which the cancer failed to grow. It reached as high titer in the animals of the tenth successive group to which the tumor was transferred (by the implantation of small bits of large neo-

plastic masses[3]) as in the rabbit of the 1st Tumor Gen., tested 15 months previously. Since the neutralizing antibody is strictly specific for the virus (6) and is identical with the complement-fixing antibody (14), the conclusion seems inescapable that the virus or an agent nearly related to it was contained in the cancer and increased in amount as the latter proliferated in host after host.

The antibody is known to have no effect upon the causative virus contained in growing papillomas, these taking their course irrespective of its titer, and the virus increasing in association with them as they do so (5). The same facts evidently hold true for the transplantable cancer and the agent it contains. In animal after animal the tumor enlarged progressively although nourished by blood containing antiviral antibody in high titer; and on every transplantation it carried with it, undiminished in antigenicity, the agent calling forth this antibody,[4]—as blood tests of the new hosts have shown.

Rabbits carrying virus-induced papillomas differ widely in their response to the presence of the growth. Immunity to the virus, as measured in terms of the neutralizing power of the blood, becomes marked in most of them within a couple of months, but in some individuals it appears but slowly and may long remain ineffectual. Occasionally a rabbit develops papillomas on reinoculation although it has carried large growths for many weeks (2),—clear evidence that it has acquired no resistance worth the name. One of the animals of Table VI (rabbit 5-86), with four big papillomas 154 days old, had no immunity demonstrable by the complement fixation test. Instances of similar kind might have been expected amongst the animals carrying the transplantable cancer, but they were not encountered: every one of 44 individuals in which the cancer had reached a large size eventually yielded serum with marked neutralizing power, and animals with small tumors and blood devoid of this power acquired it as their tumors grew.

In a previous paper (5) we have discussed some presumptive causes for the great individual differences in the immunity elicited by virus papillomas. Living cells are

[3] The active virus cannot be diluted far without loss of pathogenicity: it gives but few growths at dilutions of more than 1:10,000,—in terms of weight of papillomatous tissue extracted,—and never yields any when diluted to 1:1,000,000, even though rubbed into a very large scarified area (5).

[4] The blood of rabbit 10-40 of the 10th Tumor Gen. was found to possess marked neutralizing power on the 47th day (Table VII) and its tumors were not utilized for further transfer until the 91st day. The resulting growths in the animals of the 11th Gen. elicited antiviral antibody in the usual high titer, as proven by complement fixation tests.

known to protect viruses in general from circulating antibodies (19),—a fact which explains the continued growth of papillomas in hosts with antibody circulating in high titer. It seems reasonable to suppose that virus associated with proliferating cells may sometimes not be liberated in quantity into the organism. However this may be, it is certain that cutaneous papillomas keratinize outwards, and that in proportion as this happens the virus associated with the differentiating cells is removed from the body. It is known too that the response of an animal to the presence of a virus is largely conditioned by how much of the latter gains access to the organism. In animals carrying papillomas this amount is often far less than that which elicits a maximum immunity response, as has been shown by the success of recent experiments to increase the neutralization titer of their blood by the intraperitoneal injection of papilloma tissue procured from other rabbits (20). The titer can be greatly stepped up in this way.[5]

The conditions are significantly different in the case of the transplantable cancer. The cells of this growth are constantly dying in large numbers amidst a reactive connective tissue. The papilloma virus is markedly resistant to autolytic processes, as we have found in experiments directed to the point. It follows that the opportunities for the organism to become immunized against the virus (or an agent related to it) should be especially favorable when they carry cancers that contain it. This may be one reason why the antiviral antibody so consistently appears in the blood of cancer rabbits and reaches a high titer.

What is the papilloma virus doing in the cancer, if anything? The question has the greater interest because a distinctive substance eliciting immunity responses on the part of the host, and having chemical and physical attributes closely resembling those of the papilloma virus, has recently been found in the Brown-Pearce rabbit carcinoma (21). True, this substance will not engender growths when extracted from the carcinoma. But extracts of our transplantable cancer produced none, and the papilloma virus itself can seldom be recovered in pathogenic state from the vigorous papillomas which it directly produces in domestic rabbits and of which it is known to be the actuating cause.

In an accompanying paper we have reviewed the literature on the extraneous viruses which have been shown to flourish in tumors (22). Not a few of them persist and increase in the neoplastic tissue after the host animal has developed a resistance sufficient to prevent them from establishing themselves elsewhere in its body on reinoculation. But these viruses are in general mere "riders," producing no significant alterations in the tumor. The papilloma virus constitutes a significant exception to the rule. Not only is it capable of infecting benign and malignant growths due to some other cause, namely, the tar tumors of rabbits, but it endows some of them with the ability to grow when implanted elsewhere in the

[5] It may be remarked in passing that the papillomas of the hyperimmunized animals continued to enlarge.

host, stimulates many to more rapid proliferation, alters the morphology of not a few, and makes some become cancers forthwith instead of remaining benign and ultimately vanishing (11, 22). The growths which it influences are epidermal papillomas and squamous cell carcinomas, that is to say, tumors deriving from cells of the kind that the virus habitually acts upon, and nearly like those for which it is directly or secondarily responsible. In view of its ability to stimulate and alter these growths to which it bears no causal relationship, there would seem to be a strong likelihood that it influences the squamous cell carcinomas arising from the papillomas it has itself engendered. Yet it might exert both a stimulating and an alterative effect upon them and still not be their actuating principle. In several previous papers we have brought evidence that the change of virus papillomas to cancers is the outcome of virus variation (23, 4).

Variant alterations of viruses are usually attended by alterations in their antigenic character, one result being that animals immunized by means of the variant may respond with a greater or less immunity to the parent virus than when this is employed. The serum of the cancer rabbits of the present work neutralized the papilloma virus as well, or even better than that of animals carrying papillomas; and the immunity thus expressed developed with greater regularity. But the conditions already discussed as determining the amount of antigen set free in the body may account for this finding.

The rabbit carcinoma is now growing rapidly in eight out of twelve agouti animals of the 14th Tumor Gen. For convenience sake it will be called "Carcinoma V,2", with "V1" to designate the previous cancer of similar origin that was lost after a first transfer. The ease with which it can be maintained and the rapidity of its proliferation make it a useful experimental material, aside from its interest as a cancer of undetermined cause containing in masked or altered form the neoplastic virus concerned in its origin.

SUMMARY

A squamous cell carcinoma derived from a virus-induced rabbit papilloma has been propagated in fourteen successive groups of animals. It grows rapidly now in most individuals to which it is transplanted, killing early and metastasizing frequently. The original cancer was the outcome of alterations in epidermal cells already rendered neoplastic by the virus, and the latter, or an agent nearly related to it, has persisted and increased in the malignant tissue, as a study of the blood of the first ten groups of

cancerous animals has shown. An antibody capable of specifically neutralizing the virus *in vitro* appeared in the blood of every new host in which the tumor enlarged progressively, and reached a titer comparable with that obtaining in animals which had long carried large papillomas. The antibody was absent from normal rabbits and those in which the cancer failed to grow.

The implications of these facts are considered.

BIBLIOGRAPHY

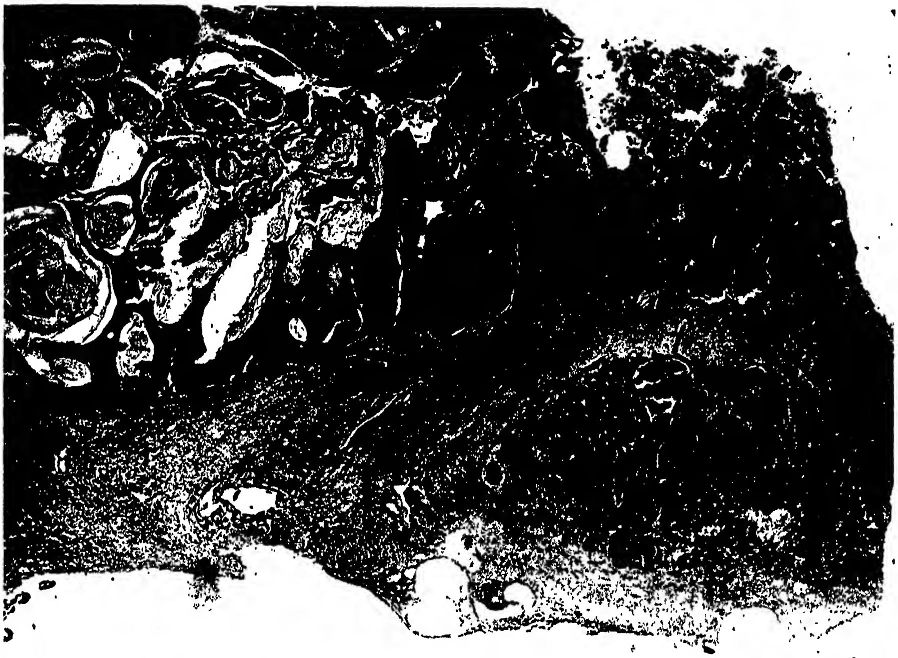
1. Syverton, J. T., and Berry, G. P., *Proc. Soc. Exp. Biol. and Med.*, 1935, **33**, 399. Rous, P., Kidd, J. G., and Beard, J. W., *J. Exp. Med.*, 1936, **64**, 385. Kidd, J. G., and Rous, P., *J. Exp. Med.*, 1940, **71**, 469.
2. Shope, R. E., *J. Exp. Med.*, 1933, **58**, 607.
3. Beard, J. W., and Rous, P., *Proc. Soc. Exp. Biol. and Med.*, 1935, **33**, 191.
4. Kidd, J. G., and Rous, P., *J. Exp. Med.*, 1940, **71**, 469.
5. Kidd, J. G., Beard, J. W., and Rous, P., *J. Exp. Med.*, 1936, **64**, 63.
6. Kidd, J. G., Beard, J. W., and Rous, P., *J. Exp. Med.*, 1936, **64**, 79.
7. Rous, P., and Beard, J. W., *J. Exp. Med.*, 1935, **62**, 523.
8. Greene, H. S. N., and Saxton, J. A., Jr., *J. Exp. Med.*, 1938, **67**, 691. Greene, H. S. N., *Science*, 1938, **88**, 357.
9. Castle, W. E., and Sawin, P. B., *Carnegie Institution of Washington, Pub. No. 427*, 1932.
10. Rous, P., Beard, J. W., and Kidd, J. G., *J. Exp. Med.*, 1936, **64**, 401.
11. Rous, P., and Kidd, J. G., *J. Exp. Med.*, 1938, **67**, 399; **68**, 529.
12. Kidd, J. G., and Rous, P., *J. Exp. Med.*, 1938, **68**, 529.
13. Kidd, J. G., *J. Exp. Med.*, 1938, **68**, 703.
14. Kidd, J. G., *J. Exp. Med.*, 1938, **68**, 703, 725, 737.
15. Brown, W. H., and Pearce, L., *J. Exp. Med.*, 1923, **37**, 601.
16. Rous, P., and Kidd, J. G., *J. Exp. Med.*, 1939, **69**, 399.
17. Rous, P., and Lange, L. B., *J. Exp. Med.*, 1914, **20**, 413.
18. Tyzzer, E. E., *J. Med. Research*, 1909, **21**, 519.
19. Rous, P., *J. Exp. Med.*, 1913, **18**, 416. Fischer, A., *Z. Krebsforsch.*, 1927, **24**, 580. Rous, P., McMaster, P. D., and Hudack, S. S., *J. Exp. Med.*, 1935, **61**, 657.
20. Kidd, J. G., *Proc. Soc. Exp. Biol. and Med.*, 1940, **43**, 770.
21. Kidd, J. G., *J. Exp. Med.*, 1940, **71**, 335, 351.
22. Rous, P., and Kidd, J. G., *J. Exp. Med.*, 1940, **71**, 787.
23. Rous, P., *Am. J. Cancer*, 1936, **28**, 233.

EXPLANATION OF PLATES

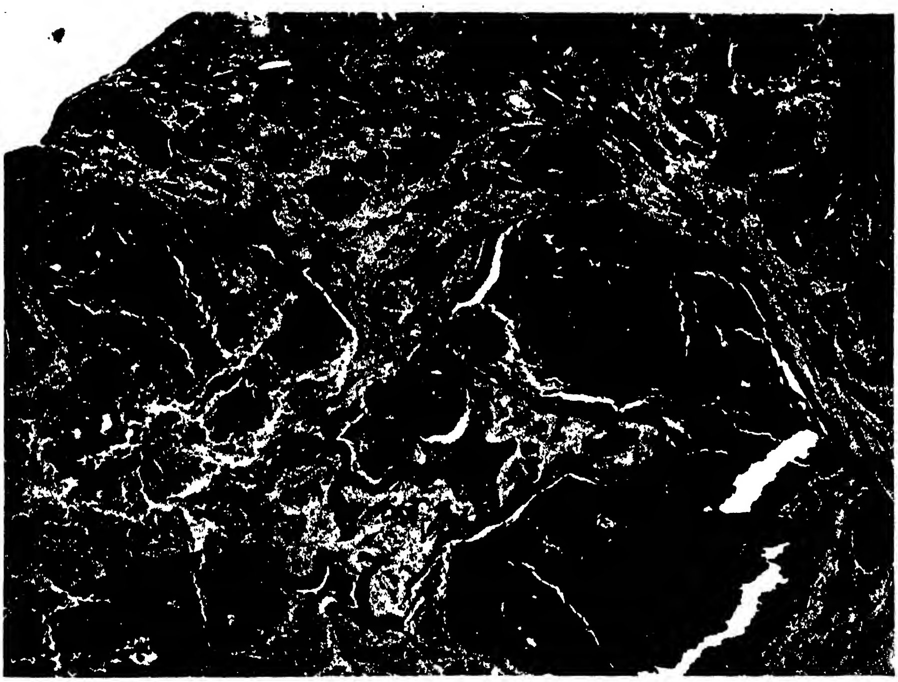
PLATE 42

FIG. 1. Radial section of one of the tumor masses on the side of the rabbit originally inoculated with papilloma virus,—to show the squamous cell carcinoma from which the metastases of Fig. 2 presumably derived. At the extreme left of the photograph normal skin with hair follicles can be seen, with keratinizing virus papilloma of the ordinary sort next it. Then comes malignant papilloma breaking up at two spots along the base into squamous cell carcinoma. At the extreme right (the center of the tumor mass) this latter has extended deep into the subcutaneous tissue. It is markedly desmoplastic, and small cysts have formed as result of early necrosis of the malignant cells. × 6½.

FIG. 2. Part of the metastatic growth in a regional lymph node, which was transferred to the leg muscles of the original host. The carcinoma shows the same features as the primary cancer. × 20.



1



2

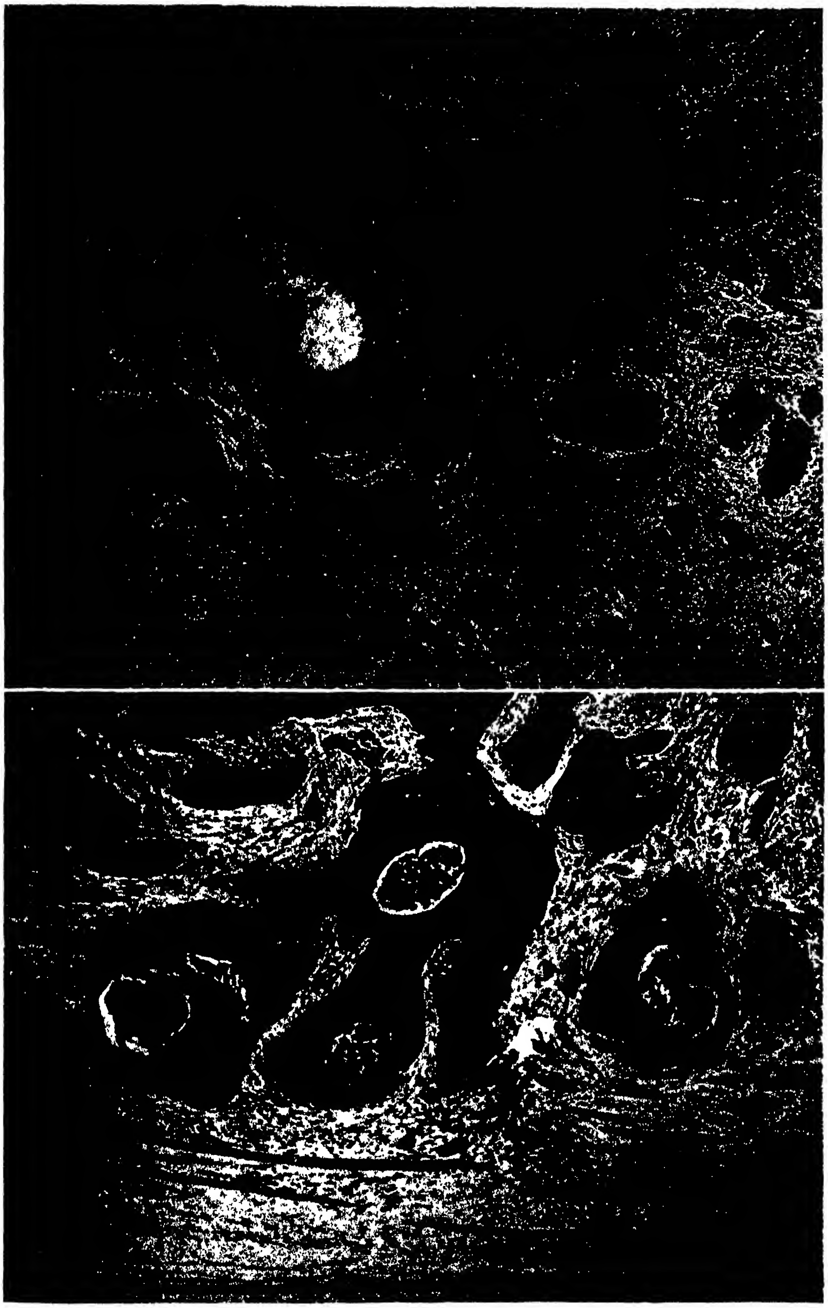
Photographed by Joseph B. Haulenbeek

(Kidd and Rous: Transplantable rabbit carcinoma containing virus)

PLATE 43

FIG. 3. Part of one of the autotransplants in the leg muscles, which furnished the tissue that was implanted in other individuals. It shows an early stage in cyst formation. $\times$ 55.

FIG. 4. Margin of a growth in the leg muscles of a rabbit of the 6th Tumor Gen. The cancer has retained its initial character and again cysts are forming. Beyond the region of reactive connective tissue proliferation edema has forced the muscle fibers apart. $\times$ 57.



Photographed by Joseph B. Haulenbeck

(Kidd and Rous: Transplantable rabbit carcinoma containing virus)

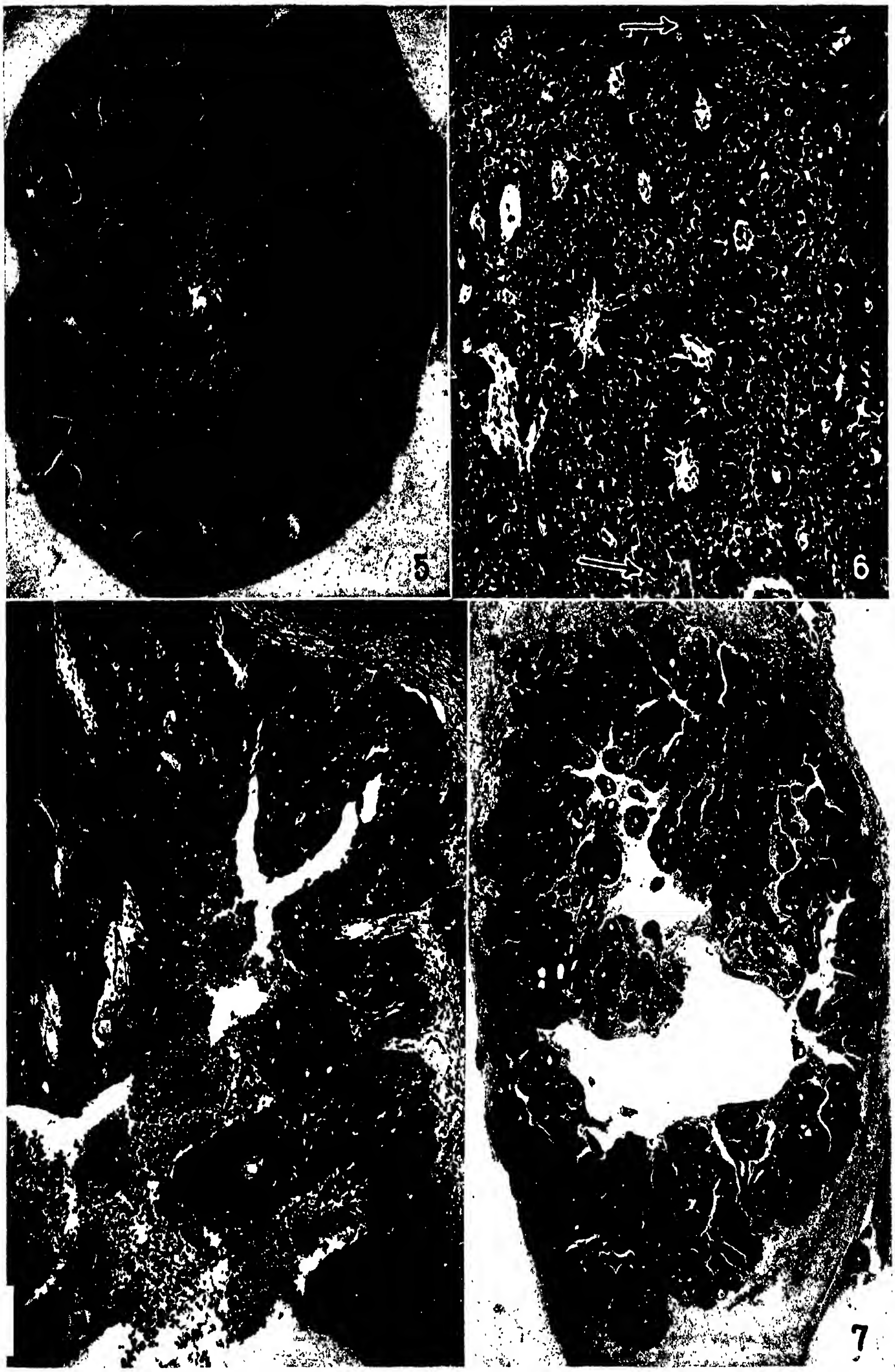
Plate 44

Fig. 5. Cross-section of an intramuscular growth 2 cm. in diameter from an animal of the 5th Tumor Gen. A,—to show the coarse pattern of the cancer, beginning cyst formation, and encapsulation. There is more reactive connective tissue than usual, and hence the growth is still solid. Compare with Fig. 7. × 4.

Fig. 6. Part of a broad expanse of cancerous cells from a rabbit of the 6th Tumor Gen. B. The malignant elements immediately next the capillaries threading the tissue are crowded and radial, while further off they have begun to die (arrows). The edge of a small cyst containing debris can be seen beyond the beginning necrosis to which the lower arrow points. × 118.

Fig. 7. Cancerous nodule from an animal of the 5th Tumor Gen. A,—to illustrate the formation of papillae by ischemic necrosis. There is much less stroma than usual and the breakdown of the cancer is correspondingly extensive. As result of it a central cyst has formed, with numerous mural papillae. These have come into being as result of the necrosis of the cancer cells furthest from the blood stream. Their cores consist of blood vessels and they are covered with a thick layer of living cancerous elements. Toward the center of the cyst the papillae are dying. × 5½.

Fig. 8. A later stage in cyst formation. The papillae covered with cancerous cells have been reduced to mere blunt protrusions as result of interior pressure and continuing necrosis. Specimen procured at the second operation on rabbit F 5-82 of the 2nd Tumor Gen. (see also Fig. 17). × 52.



Photographed by Joseph B. Haulenbeek

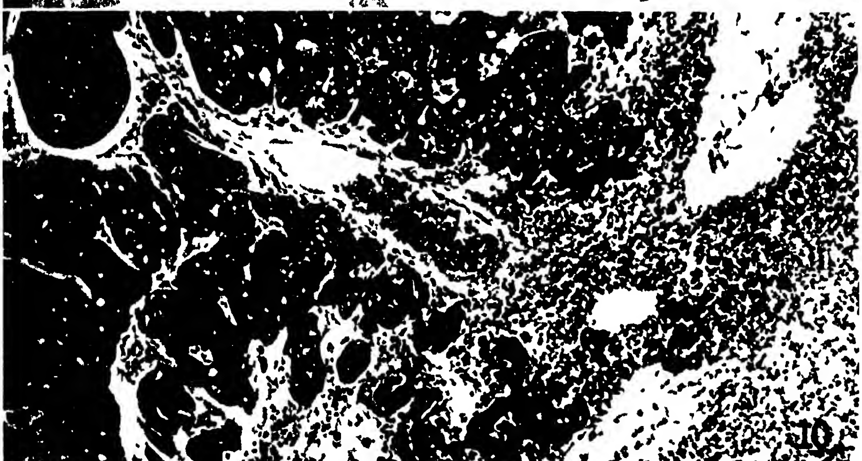
(Kidd and Rous: Transplantable rabbit carcinoma containing virus)

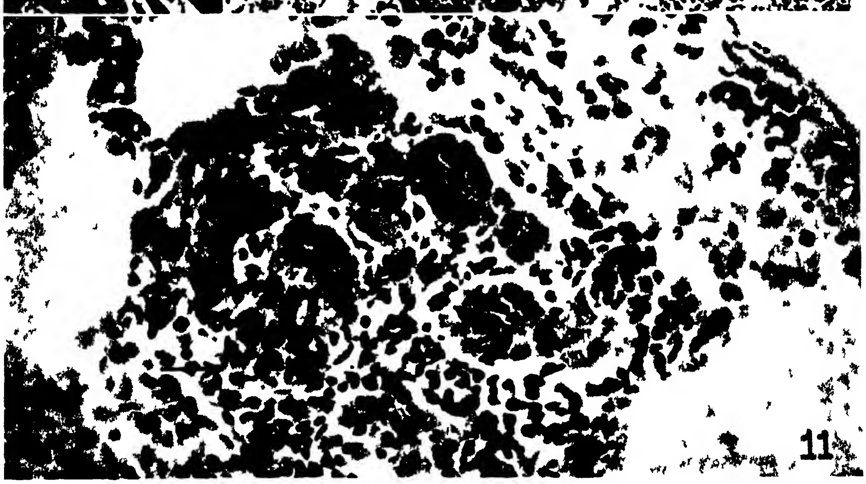
Plate 45

Fig. 9. Metastases in the iliac glands of a rabbit of the 6th Gen. B. Two glands have been entirely replaced by cancer and the process has been nearly completed in a third. The center of each growth has broken down, with result in a cyst with cancerous lining. × 6½.

Fig. 10. Part of the specimen of Fig. 9 at higher magnification,—to show direct invasion of the glandular tissue and the absence of connective tissue reaction. × 100.

Fig. 11. Pulmonary metastasis in an animal of the 8th Tumor Gen. B. There were secondaries in the axillary and iliac glands as well. The arrow points to a cell in mitotic division. × 112.





Photographed by Joseph B Haulenbeek

(Kidd and Rous Transplantable rabbit carcinoma containing virus)

Plate 46

Figs. 12 to 16. Gross changes as the tumor enlarges (see text).

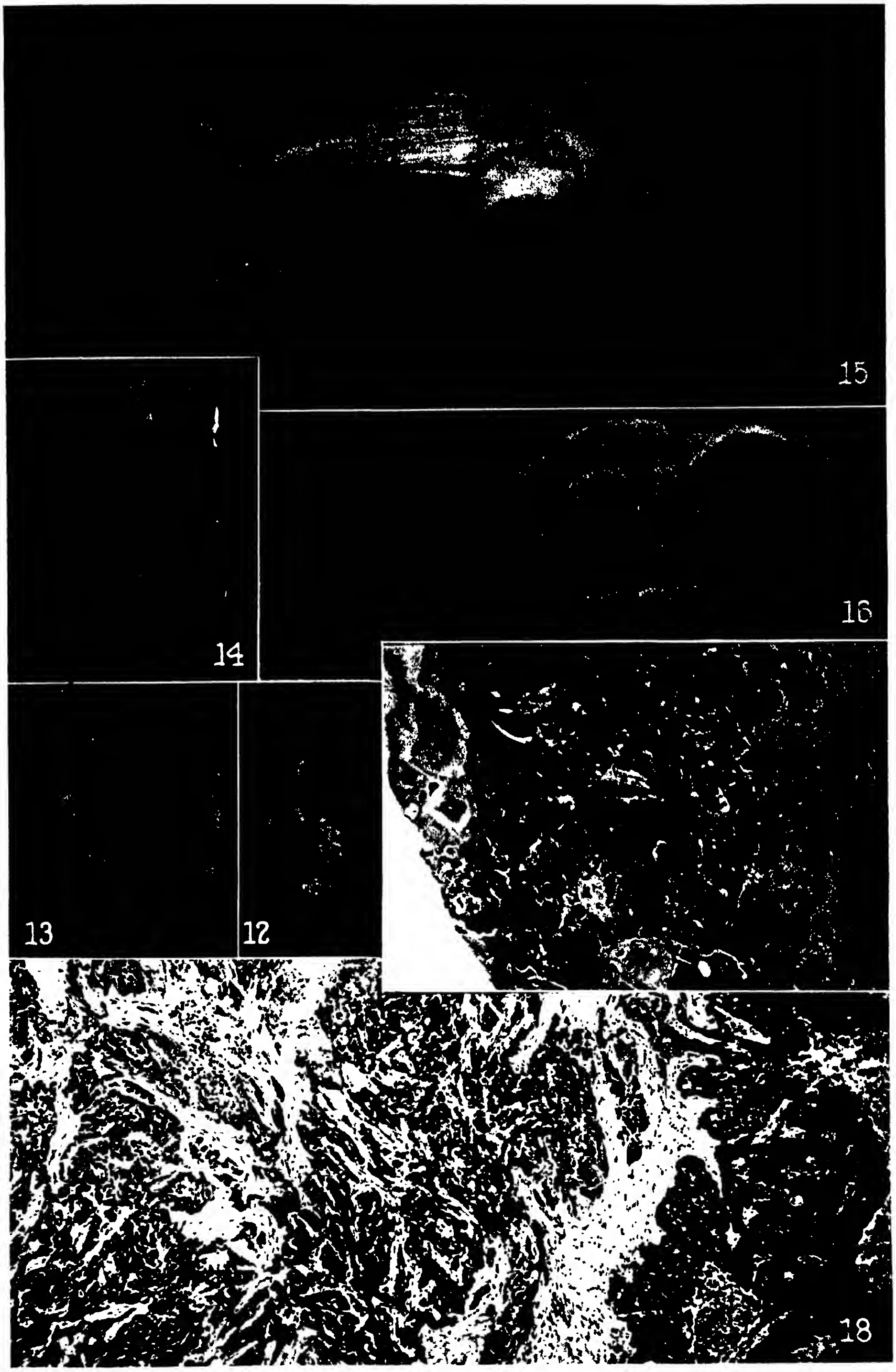
The rabbit of Fig. 15 was of the 5th Tumor Gen. A, and died of the cancer 89 days after implantation. The mass in the muscles of the right foreleg consisted of several cystic nodules, one of them about to herniate into the subcutaneous tissue. At each of the other situations there was a single, large, cystic growth. That one in the left posterior thigh was enormous.

Fig. 16, -from an animal of the 3rd Gen. killed when moribund on the 162nd day,— shows cystic tumors in the anterior and posterior thigh, emptied of their contents.

Figs. 12, 13, and 14 are natural size; Figs. 15 and 16, × ¼ and × ⅓, respectively.

Fig. 17. To show the alterations in the morphology of the cancer of rabbit F 5-82, consequent upon bacterial infection. For the previous character of the growth see Fig. 8. × 55.

Fig. 18. The extreme anaplasia found now and again in tumors of the later generations. Cancer of the ordinary type is also present, but there is no gradation from one form to the other. × 91.



Photographed by Joseph B. Haulenbeek

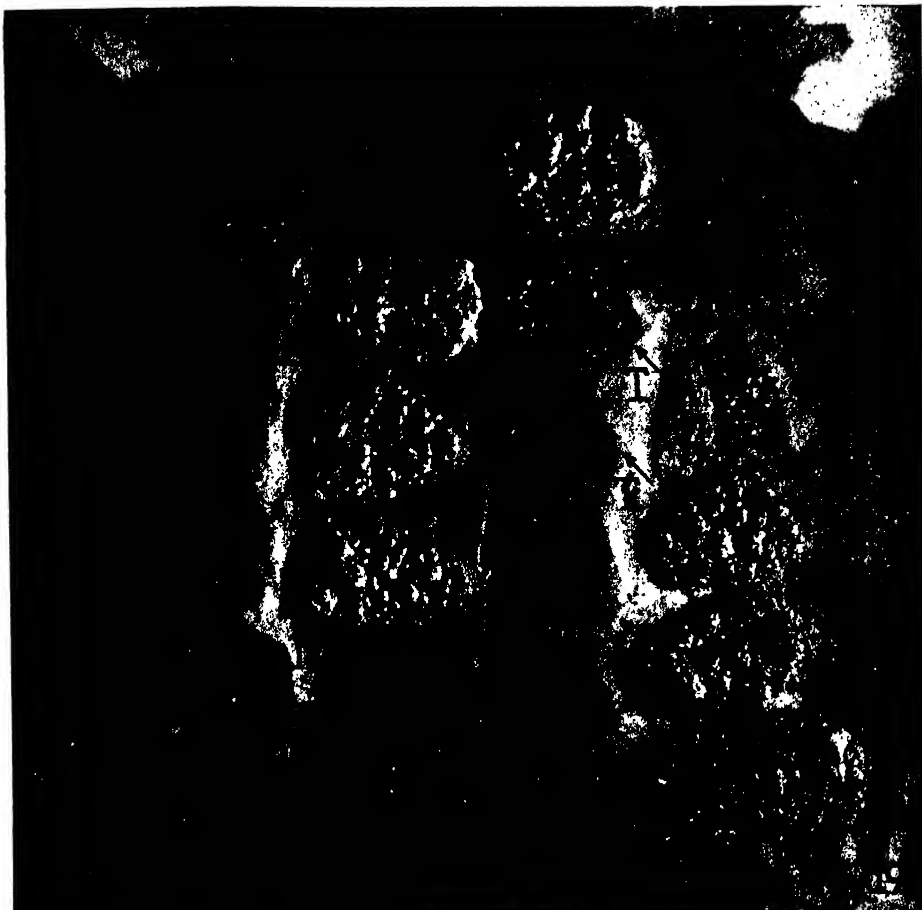
(Kidd and Rous: Transplantable rabbit carcinoma containing virus)

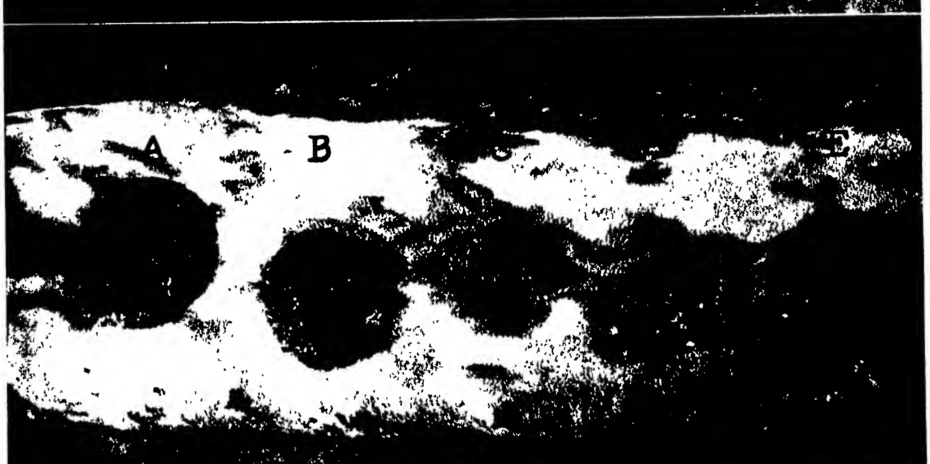
PLATE 47

FIGS. 19 and 20. The decisive character of the neutralization tests.

Fig. 19 shows the growths on the skin of test rabbit C of Table V (*q.v.*), which was killed for demonstration purposes on the 27th day after inoculation with 22 test mixtures. The skin has been dissected off and spread flat, and the strips of fur separating the inoculated areas have been shaved away. Where no growths cover the latter slight differences in their hue enable their extent to be discerned. At Tyr is an area completely covered by a confluent, papillomatous mass resulting from the inoculation of a virus-Tyrode mixture; and similar masses cover three areas (C) where mixtures of virus with the serum of 3 normal rabbits had been rubbed into the scarified skin. Five areas (P) were inoculated with mixtures containing sera from as many animals carrying large papillomas. Two of these sera prevented the virus from producing any growths at the inoculation sites, while in the other instances it caused very few. Five of the nine mixtures with the sera of cancerous animals (T) yielded no growths, two others caused only one or two, but in two instances a few more appeared than in the case of mixtures with the sera from papillomatous animals. For a record of the findings see Table V. × ½.

Fig. 20 shows five areas on the side of test rabbit A of Table I, 30 days after inoculation. Areas A and B had received mixtures of virus with serum specimens from 2 animals (5-46, 5-50) in which the cancer failed to grow after implantation. These areas are now covered with confluent papillomatosis. So too is area E which was inoculated with a mixture containing serum from a rabbit (5-52) in which a single nodule 4 mm. across appeared. At D a mixture was inoculated which contained serum from an individual (5-44) with three 4 mm. nodules in its leg muscles. A few growths have resulted. But there are none at all at site C where the mixture containing serum from rabbit 5-42, which had big tumors, was introduced. × ⅓.





Photographed by Joseph B. Haulenbeek

(Kidd and Rous: Transplantable rabbit carcinoma containing virus)

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IDENTITY OF "INHIBITOR" AND ANTIBODY IN EXTRACTS OF VIRUS-INDUCED RABBIT PAPILLOMAS

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Extracts of the growths experimentally induced in domestic rabbits with the papilloma virus often contain something that neutralizes or "inhibits" the virus when mixed with it *in vitro*, as Shope first noted (1). The nature of this phenomenon has not been determined. The present paper reports a study of the "inhibitor" and especially of its relationship to the specific antiviral antibody in the blood of animals carrying papillomas. It will be shown that the two are identical.

General Method

To test for inhibitor, the virus-induced papillomas were washed with soap and rinsed well with tap water to remove any adherent blood or virus and were then removed from the animal, using aseptic technique. The connective tissue was carefully trimmed away from the base of the growths and these were cut into small pieces and washed in isotonic saline to remove any adherent blood. Extracts of the freshly procured papillomas thus got were prepared by grinding weighed portions in sand, suspending 1:10 or 1:20 in saline (0.9 per cent), and centrifuging at about 4400 R.P.M. for 20 minutes in an International centrifuge with 51° angle head. The supernatant fluids, which were almost water-clear, were mixed in equal parts with a test virus consisting of a Berkefeld filtrate of naturally occurring cottontail rabbit papillomas. The mixtures, along with appropriate controls containing saline instead of extract, were incubated 2 hours at 37°C. and then rubbed into scarified areas on the abdominal skin of normal domestic rabbits, according to a titration technique already described (2). A number of extracts could be tested concurrently by this method. Three rabbits were used in each titration and the character of the growths was recorded at frequent intervals from the 14th to the 42nd day after inoculation of the test mixtures according to a standard scale: **** = confluent papillomatosis, *** = semiconfluent papillomatosis, ** = many discrete papillomas, * = a few discrete papillomas, ± = 3, 4, or 5 papillomas, = one papilloma, 0 = negative (complete neutralization). A comparison of the number of growths produced by the experimental mixtures with those produced by the saline controls denotes the presence or absence of inhibitor and its relative amount. When papilloma extracts containing no inhibitor were mixed with the test virus and inoculated into normal rabbits, the resulting yield of growths was like that produced by the virus-saline control inoculum; whereas fewer or none were got when extracts containing inhibitor had partially or completely neutralized the virus. The inhibitor in a given extract could be titrated by testing the capacity of various dilutions of it to neutralize the test virus.

The *test viruses* were prepared by grinding weighed portions of naturally occurring cottontail rabbit papillomas, which had been preserved in 50 per cent glycerol, suspending the material in 10 or 20 volumes of saline, and centrifuging at about 4400 R.P.M. for 20 minutes in the angle centrifuge. The supernatant fluids were then filtered through Berkefeld V candles.

Blood was obtained from rabbits bearing the papillomas by bleeding from an ear vein or by cardiac puncture. The serum was removed after clotting and tested for antibody by means of the neutralization and complement fixation tests devised in this laboratory, both of which have been described (2, 3). In the *neutralization test* various dilutions of serum were mixed in equal parts with a test virus and put into a water bath at 37°C. for 2 hours. The mixtures were then rubbed into scarified skin areas of normal domestic rabbits, and the character of the growths was recorded by asterisks according to the standard scale described above for the detection of inhibitor in tissue extracts. In the *complement fixation test* various dilutions of serum were tested in mixture with 2 units of complement (titrated immediately beforehand) and an optimal amount of a test virus as the antigen. The mixtures stood at room temperature for 2 hours to allow fixation of the complement and then sensitized red cells were added. Readings were made after 30 minutes in a water bath at 37°C. and again after the tubes had stood overnight in a refrigerator. The latter readings are recorded in the tables as follows, in terms of fixation: ++++ = complete fixation (no hemolysis), +++ = about 75 per cent fixation, ++ = about 50 per cent fixation, + = about 25 per cent fixation, ± = about 10 per cent fixation, 0 = no fixation (complete hemolysis). It has been shown that the virus-neutralizing and complement-fixing capacities of any given serum invariably parallel one another (3, 4). The neutralization test has a slightly lower threshold for the detection of the antibody than the complement fixation test. The latter, however, gives immediate and reliable quantitative results when any considerable quantity of antibody is present, and hence it has been largely used in the present experiments.

It will be noted that the asterisks in all the tables record the number of papillomas produced upon inoculation of the virus in mixture with serum, tissue extracts, or saline. Hence neutralization or inhibition of the virus is demonstrated by fewer papillomas recorded for the experimental mixtures as compared with the controls. The plus signs on the other hand record the effectiveness of the blood antibody, as indicated by its capacity to fix complement in mixture with the virus.

D. R. means domestic rabbit; W. R., wild cottontail rabbit.

Yield of Inhibitor from Papillomas of Rabbits with Different Amounts of Antibody in Their Blood

As a first step toward learning whether there is a relationship between inhibitor and antibody, comparative tests were made of the yield of inhibitor from the papillomas of domestic rabbits and the amount of antibody in the sera of the animals. It was already known that the blood of rabbits carrying such growths acquires in most instances the capacity to neutralize the papilloma virus (2) and to fix complement when mixed with it *in vitro* (3).

Experiment 1.—A 5 per cent virus filtrate of the naturally occurring glycerolated papillomas of W. R. 1-10 was rubbed into several scarified skin areas, each about 4 cm.

square, on the abdomens of six normal domestic rabbits. Scarification had been done with sterilized sandpaper. On the 70th day after inoculation each rabbit had large papillomatous masses covering the areas. They were now bled from an ear vein for serum, and under ether anesthesia a papilloma from each rabbit was removed and a 10 per cent saline extract of it prepared according to the method described in the preceding section. The extracts were then tested for capacity to neutralize a 5 per cent virus filtrate (W. R. 1-10), using the standard technique and inoculating the mixtures into

TABLE I

Yield of Inhibitor from the Papillomas of Domestic Rabbits with Differing Amounts of Blood Antibody

Papillomas from rabbit No.	Tests for inhibitor: Growths resulting from mixture of virus and papilloma extract†			Serum antibody titer: Complement fixation tests‡: Dilutions of serum						
	a	b	c	1:4	1:8	1:12	1:16	1:24	1:32	1:48
1	***̲	***	***̲	++++	0	0	0	0	0	0
6	*4*̲	**̲	**	++++	++++	±	0	0	0	0
5	*	*̲	*	++++	++++	++++	+++±	±	0	0
3	0	0	*̲	++++	++++	++++	++++	+++	++	0
2	*̲	*̳	*̳	++++	++++	++++	++++	++++	+++±	+±
4	*̲	*	*̲	++++	++++	++++	++++	++++	++++	++++
Virus filtrate plus Tyrode (controls).	****̲	****	****							

† Inoculum = 5 per cent virus filtrate (W. R. 1-10) and 10 per cent papilloma extracts mixed in equal parts.

**** = confluent papillomatosis.
*** = semiconfluent papillomatosis.
** = many discrete papillomas.
* = a few " "
*̲ = 3, 4, or 5 papillomas.
*̳ = one papilloma.
0 = negative.

Growths in test rabbits (a, b, c) on the 42nd day after inoculation.

‡ Antigen, W. R. 1-10 virus filtrate, 1:60. Complement, 2 units in all tubes.

++++ = complete fixation (no hemolysis).
+++ = about 75 per cent fixation.
++ = " 50 " " "
+ = " 25 " " "
± = " 10 " " "
0 = no fixation (complete hemolysis).

scarified skin areas of three normal domestic rabbits. The serum of each rabbit was tested in various dilutions for antibody by the complement fixation test, using the method described. The W. R. 1-10 virus filtrate diluted 1:60 in saline served as the antigen.

The results of these tests are shown in Table I. The papillomas from D. R. 2, 3, 4, and 5 yielded an inhibitor so potent as to neutralize the test virus almost completely, whereas extracts of the growths of the remaining two rabbits (D. R. 1 and 6) had only a slightly adverse effect on the virus. The antibody titers of the sera of these rabbits varied widely, and on comparison it will be seen that those rabbits in which the titer of serum antibody was most pronounced (D. R. 2, 3, 4, 5) were those with papillomas yielding most inhibitor.

To study further the relation between the inhibitor and serum antibody, papillomas were produced with the virus in another group of domestic rabbits and portions of the growths were removed from time to time and tested for their content of inhibitor, the amount of antibody in the sera of the rabbits being determined concurrently.

Experiment 2.—A 5 per cent virus filtrate of the naturally occurring glycerolated papillomas of W. R. 1-10 was rubbed into a scarified area about 4 cm. square and tattooed as well into eight small areas on the abdomens of six normal domestic rabbits. On the *21st day* after inoculation the rabbits had discrete and semiconfluent papillomatous masses covering the tattoo and scarified inoculation areas, respectively. Each was bled about 10 cc. for serum from an ear vein, and under ether anesthesia a wedge of the semiconfluent papillomatous tissue and several of the discrete growths were removed from each with different sets of sterile instruments.[1] The gaps in the growths were closed with sutures and healing occurred without infection. 10 per cent saline extracts of the excised tissue were prepared in the usual manner and tested for inhibitor in dilutions of 1:10 and 1:20, mixing them with equal parts of a 5 per cent virus filtrate of the infectious papillomas of W. R. 1-10. This virus filtrate was kept frozen at $-70°C$. and used throughout this experiment. It underwent no detectable decrease in infectivity when inoculated in various dilutions into test rabbits after being frozen 9 months, the longest period employed. After incubating 2 hours at 37°C. the mixtures were rubbed into scarified skin areas of three normal domestic rabbits. The serum of each rabbit was tested in dilutions of 1:10 and 1:20 for capacity to neutralize the virus filtrate and in addition the titer of each serum was determined by complement fixation tests, using another virus filtrate (W. R. 1-56) diluted 1:120 in saline as the antigen.

On the *37th day* another representative portion of the large growth of each rabbit was removed and tested for inhibitor precisely as before. Just before the excision each rabbit was bled from an ear vein and the antibody titers of the sera again determined by the neutralization and complement fixation tests. One rabbit had died and the growths of another had retrogressed during the 4th week after inoculation; hence the results with only four rabbits are available.

On the *70th day* the sera of the rabbits were tested again for antibody content, using

[1] The tests with the discrete papillomas will not be considered in detail. It was found that the yield of inhibitor from discrete and confluent papillomas of the same rabbit was not appreciably different in this experiment and in other tests. In a few instances, however, the discrete papillomas yielded significantly less inhibitor than did the confluent growths, suggesting that the latter afforded a greater opportunity for the extravasation of blood antibody, as Kidd found to be the case regularly in the confluent growths of cottontail rabbits (6). It should be noted that the discrete papillomas of cottontail rabbits usually form orderly, intact, onion-like cones with slightly constricted bases, whereas the discrete growths of domestic rabbits are sessile and become fissured and disorderly early in their development, resembling in this respect the large confluent papillomas of both species. It has been shown that blood antibody is more likely to extravasate in quantity into the fissured, disorderly growths of cottontail rabbits than into intact, discrete papillomas (6).

the complement fixation test. No significant change in antibody titer had taken place, and hence recourse was had to intraperitoneal injections of virus as a means of stimulating a high titer of circulating antibody. Two of the rabbits (D. R. 97 and 100) were given 5 cc. of a 5 per cent freshly prepared virus filtrate (W. R. 1-30) intraperitoneally on the 92nd and again on the 102nd day. The remaining two rabbits (D. R. 96 and 101) were not hyperimmunized.

On the *111th day* the papillomas of three of the rabbits had increased in size, had fleshy bases, and were now fissured and somewhat macerated. The growth of D. R. 100, however, was no larger and a thick connective tissue layer was present beneath it. Again a portion of each was removed, extracted, and tested for inhibitor exactly as on the 21st and 37th days, and serum was obtained just beforehand and tested for antibody as before. Microscopic sections showed that all the growths were still benign papillomas.

The results of the successive tests are summarized in Table II. On the *21st day*, when the first operation was done to procure papillomatous tissue, the serum of every rabbit had a low antibody titer. The serum from D. R. 100 had no antibody detectable either by virus neutralization or complement fixation tests, while the sera of D. R. 96, 97, and 101 partially neutralized the virus in dilutions of 1:10 and 1:20 and fixed complement in low titers. The extracts of the papillomas of these rabbits had no detectable effect upon the ability of the test virus to cause growths. On the *37th day* there were notable differences in the findings. The serum antibody titer of one of the rabbits (D. R. 100) had risen considerably and the extract of the papillomas of this rabbit markedly reduced the pathogenic activity of the virus. Only a slight rise in the antibody titer of the sera of the other rabbits had occurred and the inhibitor content of their papillomas had also increased but slightly. The results of the tests on the *111th day* were quite remarkable in that the sera from three of the rabbits (D. R. 96, 97, 100) now had high antibody titers and their papillomas yielded large amounts of the inhibitor, whereas the serum of the remaining rabbit (D. R. 101) had almost no circulating antibody and no inhibitor could be procured from its papillomas. There had been a spontaneous increase in the blood antibody of rabbit D. R. 96, with result that it now fixed complement in high titer and almost completely neutralized the virus in dilutions of 1:10 and 1:20. The papilloma extract of this rabbit also neutralized the test virus when mixed with it in dilutions of 1:10 and 1:20. Rabbit D. R. 97, which had received hyperimmunizing injections of virus intraperitoneally, now had a high serum antibody titer, and its papilloma extract neutralized the virus markedly. D. R. 100, also hyperimmunized, yielded similar results. Additional tests, not shown in the table, with the materials obtained from D. R. 97 and 100 on the 111th day, revealed that the sera of both when diluted 1:100 almost completely neutralized the virus, while the papilloma extracts, though exerting a pronounced neutralizing effect in dilution of 1:40, did little at 1:80. D. R. 101 provided a remarkable control in that its serum manifested a low titer of circulating antibody throughout these tests and its papillomas at no time yielded any significant amount of inhibitor.

The results of these experiments (Tables I and II) show that the amount of inhibitor in extracts of the papillomas of domestic rabbits and the serum antibody titer of the host vary concurrently not only from animal to animal but in individual rabbits. In an exceptional instance in which

TABLE II

Yield of Inhibitor from the Same Papillomas at Different Times, Compared with the Changes in Circulating Antibody

Time to biopsy of growths	Source of material	Yield of inhibitor				Serum antibody titer								
			Growths† resulting from mixture of 5 per cent virus filtrate (W. R. 1-10) and				Growths† resulting from mixture of 5 per cent virus filtrate (W. R. 1-10) and			Complement fixation tests§				
	Rabbit No.	Test rabbits	Saline	Dilutions of papilloma extract		Test rabbits	Saline	Dilutions of serum		Dilutions of serum				
				1:10	1:20			1:10	1:20	1:4	1:8	1:16	1:32	1:64
days														
	100	1	***±	****	****	19	****	****	****	0	0	0	0	0
		2	****	***±	***±	20	***±	***	***±					
		3	***±	***	***±	21	****	***±	****					
	97	4	***±	**±	***	19	****	±	*±	++++	±	0	0	0
		5	****	***±	****	20	***±	±	**±					
		6	****	***±	****	21	****	*	*					
21	96	1	***±	***±	****	19	****	±	±	++++	+++±	0	0	0
		2	****	***±	****	20	***±	*	**					
		3	***±	***	****	21	****	±	±					
	101	4	***±	****	****	19	****	***	***	±	0	0	0	0
		5	****	***±	***±	20	***±	*±	**					
		6	****	***	****	21	****	**	**±					
	100	7	***±	*	*±	22	****	±	*	++++	++++	++++	+	0
		8	****	±	*	23	****	0	*					
		9	***±	±	±	24	****	±	*					
	97	10	***±	**	***	22	****	*±	*±	++++	++±	0	0	0
		11	****	***	***±	23	****	±	**±					
		12	***±	**	***	24	****	*	*±					
37	96	7	***±	***	***±	22	****	*	**±	++++	+++±	0	0	0
		8	****	***±	****	23	****	*	±					
		9	***±	***	****	24	****	±	*					
	101	10	***±	***±	****	22	****	***±	***±	+++	0	0	0	0
		11	****	***	***	23	****	**	***±					
		12	***±	***±	***	24	****	***	***					
	100‡	13	***±	±	*	13	***±	0	0	++++	++++	++++	++++	++++
		14	****	±	*±	14	****	±	0					
		15	***±	0	±	15	***±	0	±					
	97‡	16	***±	±	*	16	***±	0	±	++++	++++	++++	++++	±
		17	****	±	*	17	****	0	±					
		18	****	±	±	18	****	0	±					
111	96	13	***±	*	*	13	***±	0	±	++++	++++	++++	+	0
		14	****	±	*±	14	****	0	±					
		15	***±	±	**	15	***±	±	±					
	101	16	***±	****	***±	16	***±	***	****	0	0	0	0	0
		17	****	****	****	17	****	***	****					
		18	****	****	****	18	****	**±	***±					

† Readings made on the 35th day after inoculation of the mixtures, according to the standard scale (see Table I).

‡ Received hyperimmunizing injections of virus intraperitoneally on 92nd and 102nd days.

§ Complement, 2 units in all tubes.

Antigen, W. R. 1-56 virus filtrate, 1:120.

antibody was practically lacking in the serum over a long period (D. R. 101 of Experiment 2), inhibitor could not be got from the papillomas.

The findings have been repeatedly confirmed. Papillomas of various size and duration have been tested from 36 rabbits in all. Of these 22 had high titers of serum antibody as determined by virus neutralization and complement fixation tests and their papillomas all yielded large amounts of the inhibitor, whereas little or none could be detected in similar growths from 14 domestic rabbits that had but little circulating antibody. Papillomas that had only recently arisen yielded little or none of the inhibitor, and the sera of the hosts contained little or no antibody. Later on the amount of inhibitor procurable from the growths increased in general proportion to the rise in serum antibody titer. All this indicates the existence of a relationship between the amount of antibody in the blood and the amount of inhibitor in extracts of the papillomas.

The Presence of Inhibitor in Organ Extracts of Rabbits

If the inhibitor in papilloma extracts is blood antibody, as indicated by the preceding experiments, it should also be present in non-papillomatous tissues of rabbits carrying the growths. To test this, organ extracts of normal rabbits and of rabbits carrying papillomas were tested for inhibitor. And to enlarge the observations, extracts of the Brown-Pearce rabbit carcinoma were also tested. It is known that animals carrying this tumor develop no antibodies against the papilloma virus (5).

Experiment 3.—A domestic rabbit (D. R. 1-55), that had carried several large papillomas for 34 days and had received three intraperitoneal injections of a 5 per cent virus filtrate (W. R. 1-28) at weekly intervals to stimulate a high titer of circulating antibody, was bled for serum and killed. Portions of the papillomas, skin, muscle, and liver were immediately removed and 10 per cent saline extracts of them were prepared in the usual way. The extracts and serum diluted 1:10 with saline were tested for capacity to neutralize a freshly prepared 1 per cent virus filtrate (W. R. 1-30). Another domestic rabbit (D. R. 86), with several large papillomas of about 18 weeks' duration, was also bled for serum and killed. A previous complement fixation test had shown that this rabbit had a low serum antibody titer. Portions of papilloma, skin, muscle, and liver were removed and 10 per cent saline extracts prepared. The extracts and serum were tested for capacity to neutralize another 1 per cent virus filtrate (W. R. 1-56).

Experiment 4.—Three normal domestic rabbits (D. R. 1, 2, and 3) were bled for serum under ether anesthesia and then killed. Portions of skin, muscle, and liver were immediately removed from each rabbit and 10 per cent saline extracts were prepared. The extracts and undiluted sera of two of these rabbits (D. R. 1 and 2) were tested for capacity to neutralize a 1 per cent virus filtrate (W. R. 1-56), while the extracts and serum of D. R. 3 were tested with a 5 per cent virus filtrate (W. R. 1-10). In addition 10 per cent saline extracts of the Brown-Pearce tumor prepared from muscle transplants

of two domestic rabbits (D. R. 11 and 8-90), which had been kept frozen, were tested for capacity to neutralize the same virus filtrates.

TABLE III

Yield of Inhibitor from Papillomas, Brown-Pearce Carcinomas, and Certain Organs of Domestic Rabbits

Source of material	Rabbit No.	Test rabbits	Yield of inhibitor						Serum antibody titer		
			Growths resulting from mixture† of virus filtrate and						Complement fixation tests‡		
									Dilutions of serum		
			Saline (control)	Papilloma extract	Skin extract	Liver extract	Muscle extract	Brown-Pearce tumor extract	1:4	1:16	1:64
Rabbits with papillomas	D. R. 1-55	a	****	0	*	*	***±		++++	++++	++++
		b	****	±	±	±	***				
		c	***±	±	*	±	*±				
	86	d	****	**	***	***	****		+++±	0	0
		e	***±	*	**±	**	***				
		f	****	**	**±	**	***				
Normal rabbits	1	g	****		****	****	****		0	0	0
		h	****		****	****	***±				
		i	****		****	****	****				
	2	g	****		****	****	****		0	0	0
		h	****		****	****	****				
		i	****		****	****	****				
	3	g	****		***±	****	***±		0	0	0
		h	****		****	***	****				
		i	****		****	***	****				
Rabbits with Brown-Pearce carcinoma	11	g	****					****	0	0	0
		h	****					****			
		i	****					****			
	8-90	g	****					****	0	0	0
		h	****					****			
		i	****					****			

† Inoculum = 10 per cent extracts and 1 per cent virus filtrate mixed in equal parts, except extracts from D. R. 2 and 8-90 which were tested with a 5 per cent virus filtrate.

Readings made on the 42nd day after inoculation, according to the standard scale.

‡ Complement, 2 units in all tubes.

Antigen, W. R. 1-56 virus filtrate, 1:120.

The sera of all of the rabbits used in Experiments 3 and 4 were also tested for antibody by means of the complement fixation test, using the virus filtrate (W. R. 1-56) diluted 1:120 in saline as the antigen.

The results of these tests are summarized in Table III. The serum neutralization tests gave results similar to those obtained with the comple-

ment fixation tests and hence are not included in the table. None of the inhibitor could be detected in saline extracts of the skin, muscle, and liver of the three normal rabbits, nor did extracts of the Brown-Pearce carcinoma, from rabbits uninfected with the papilloma virus, exert any effect upon the latter. The sera of these rabbits contained no antibody detectable either by neutralization or complement fixation tests. In contrast to these findings the inhibitor was present in extracts of the papillomas, skin, muscle, and liver of rabbits carrying the growths. These extracts from rabbit D. R. 1-55, which had a high serum antibody titer, contained large amounts of the inhibitory substance, while similar extracts of rabbit D. R. 86, which had little serum antibody, caused relatively slight neutralization of the virus. Organ extracts of three other rabbits bearing papillomas were also tested, and similar results were obtained. They have not been tabulated. The neutralizing effect of 10 per cent extracts of the papillomas was regularly less than that of a 10 per cent dilution of the sera, but was somewhat greater than that of extracts of the skin, muscle, and liver from the same rabbit. Further tests showed that hemolysin injected intravenously into rabbits carrying papillomas could be detected in extracts of the growths and of normal tissues (skin, muscle, liver). Conditions within papillomas are evidently favorable to the passage of antibody into them.

The Presence of Inhibitor in Cottontail Papillomas

The papilloma virus can usually be procured from the growths of cottontail rabbits, but far from always, and Kidd has shown (6) that the failure to recover it is due to the extravasation of blood antibody into them. Will they on extraction yield the inhibitor, like the papillomas of domestic rabbits? To learn about this, large, vigorously growing papillomas were produced in normal cottontail rabbits by inoculating the virus into broadly scarified areas on the skin of the abdomen. The animals were also injected intraperitoneally with a suspension of the papilloma virus to stimulate a high titer of circulating antibody. For it was known that "masking" of the virus is most likely to occur in the large growths of cottontails having much antibody in their blood (6).

Experiment 5.—A 10 per cent extract of the glycerolated papillomas of W. R. 1-28 was prepared by grinding in sand, suspending in saline, and centrifuging at 2500 R.P.M. for 5 minutes in the angle centrifuge. Some of the turbid supernatant fluid was rubbed into a large scarified area on the abdomen of each of four normal cottontail rabbits. The remainder was again spun at 4400 R.P.M. for 20 minutes, the supernatant fluid was filtered through Berkefeld V candles and diluted in saline to make a 5 per cent suspension; and 6 cc. of the filtrate per kilogram body weight was injected intraperitoneally into

each rabbit on the day after skin inoculation and again on the 8th and 19th days. Complement fixation tests showed that even before the papillomas appeared these rabbits had high serum antibody titers. This state of affairs had no apparent influence on the course of the growths. They enlarged as rapidly as usual.

On the 29th day after skin inoculation, two of the rabbits (W. R. 24 and 27) were bled for serum tests and killed. Each had a large, fleshy papilloma. 10 per cent saline extracts of the growths were immediately made and tested, as were the sera, for capacity to neutralize a 1 per cent virus filtrate (W. R. 1-30). The extracts were also tested for infectiousness by inoculating them into the scarified skin of three normal rabbits.

On the 71st day the remaining rabbits (W. R. 22 and 26) had still larger papillomatous masses, now deeply fissured, with dry, keratinized tops and fleshy bases. Each was bled for serum and killed and 10 per cent extracts were made of the growths. In addition 10 per cent extracts of the skin, muscle, and liver of W. R. 26 were prepared. The extracts and sera were tested for capacity to neutralize the same 1 per cent virus filtrate (W. R. 1-30).

10 per cent saline extracts of the skin, muscle, and liver of a normal cottontail rabbit were also prepared after it had been bled for serum. The materials were tested for capacity to neutralize a 1 per cent virus filtrate (W. R. 1-56).

In additional tests the sera of all the rabbits were tested for complement-fixing antibody.

Table IV shows the results of these tests. (The serum neutralization tests are not included in the table since they only confirmed the results obtained with the complement fixation tests.) Extracts of the growths from rabbits with high antibody titers (W. R. 27, 24, and 22) yielded no virus on inoculation into two rabbits (one of the three test rabbits died 4 days after inoculation). These extracts contained inhibitor in relatively large amount. The serum of W. R. 26 had a low antibody titer and its growth contained but a slight amount of inhibitor and yielded a small amount of virus, as evidenced by the papillomas produced.

The skin, muscle, and liver extracts of W. R. 26 also yielded a small amount of inhibitor. Extracts of the skin, muscle, and liver of the normal cottontail rabbit had no capacity whatever to neutralize the virus, and its serum contained no antibody detectable by neutralization or complement fixation tests.

The tests with cottontails having papillomas that yielded little or no active virus gave findings essentially similar to those in domestic rabbits. Inhibitor proved obtainable from the growths in amount roughly proportional to the serum antibody titer of the host. The amount of inhibitor in extracts of these papillomas was considerably less than that procured from growths in domestic rabbits with comparable blood antibody titers, presumably because of the specific absorption of inhibitor by the virus present in the cottontail growths (4).

Complement Fixation Tests for Inhibitor

The serum antibody that neutralizes the papilloma virus *in vitro* has been shown to be identical with the antibody responsible for the fixation

TABLE IV

Yield of Inhibitor from Papillomas and Certain Organs of Cottontail Rabbits

Source of material	Rabbit No.	Test rabbits	Yield of inhibitor: Growths† resulting from mixture of virus filtrate and Saline (control)	Papilloma extract	Skin extract	Liver extract	Muscle extract	Test rabbits	Yield of virus: Growths† resulting from inoculation of the papilloma extracts	Serum antibody titer: Complement fixation tests‡, Dilutions of serum 1:4	1:8	1:16	1:32	1:64	1:128
Rabbits with papillomas	27	a	***±	*				a	0	++++	++++	++++	++++	++++	++++
		b	***±	*				b	0						
	24	a	***±	±				a	0	++++	++++	++++	++±	0	0
		b	***±	*				b	0						
	22	c	****	*				l	0	++++	++++	++++	±	0	0
		d	****	*±				m	0						
		e	****	±											
	26	f	***±	***	**	**±	***	l	*	+++±	0	0	0	0	0
		g	****	***±	**±	**	***±	m	*						
		h	****	****	**	*±	***±								
Normal rabbit	1	i	****		****	****	****			0	0	0	0	0	0
		j	***±		****	****	****								
		k	****		***	****	***								

† Inoculum = 1 per cent virus filtrate (W. R. 1-30) and 10 per cent extracts mixed in equal parts.

Readings made on the 35th day after inoculation according to the standard scale.

‡ Complement, 2 units in all tubes.

Antigen, W. R. 1-56 virus filtrate, 1:120.

TABLE V

Antibody in Sera Compared with Inhibitor in Papilloma Extracts

Rabbit No.	Material	Complement fixation tests†: Dilutions of material 1:4	1:8	1:16	1:32	1:64	1:128	1:256	Neutralization tests: Growths§ resulting from mixture of 5 per cent virus filtrate (W. R. 1-68) and serum or papilloma extract, 1:4: a	b	c
D. R. 10‡	Serum	++++	++++	++++	++++	++++	+++±	±	0	0	0
	Papilloma extract	++++	++++	++++	++++	++			±	0	±
2-47‡	Serum	++++	++++	++++	++++	++++	+++±	0	±	0	0
	Papilloma extract	++++	++++	++++	+	0			±	±	±
24	Serum	+++±	0	0	0	0	0	0	±	±	±
	Papilloma extract	0	0	0	0	0			****	***	***±
2-50	Serum	0	0	0	0	0	0	0	**	***	**
	Papilloma extract	0	0	0	0	0			****	****	****
5 per cent virus filtrate plus saline (controls).........									****	****	****

† Complement, 2 units in all tubes.

Antigen, W. R. 1-72 virus extract, 1:120.

None of the materials was anticomplementary when tested in double amount.

‡ Received two hyperimmunizing injections of papilloma virus intraperitoneally.

§Growths in test rabbits (a, b, c) on 42nd day after inoculation according to the standard scale.

of complement (3, 4). Will the inhibitor in extracts of papillomas also fix complement in mixture with the virus? To answer this question, extracts of the papillomas of domestic rabbits containing various amounts of inhibitor were tested for capacity to fix complement in mixture with the papilloma virus. The sera of the rabbits furnishing the growths were tested in the same way for comparison.

Experiment 6.—Four rabbits (D. R. 10, 24, 2-47, 2-50) with papillomas of 31 to 45 days' duration were utilized. Two of them (D. R. 10 and 2-47) had received two intraperitoneal injections of a 5 per cent virus filtrate (W. R. 1-28) to raise the circulating antibody to a high titer. All were bled for serum and killed. The papillomas of each rabbit were removed, passed through several changes of saline, and 1:4 suspensions were prepared by grinding in sand and suspending in saline. The extracts were then spun at 4400 R.P.M. for 20 minutes in the angle-head centrifuge. In order to avoid the anticomplementary effect that many papilloma extracts show in suspensions of 1:10 or less, the supernatant fluid was again spun at 20,000 R.P.M. in the air-driven centrifuge and then heated at 60°C. for 30 minutes. A heavy, flocculent precipitate formed which was removed by centrifugation at 4400 R.P.M. for 20 minutes. (It had previously been determined that these procedures do not significantly affect the inhibitor content of the papilloma extracts.) The final supernatant fluids were water-clear with a faint amber color. They were not anticomplementary.

The papilloma extracts thus prepared and the sera of each rabbit were tested in various dilutions for capacity to fix complement in mixture with the papilloma virus. The technique of the test was that previously described (3), using 2 units of complement in each tube and an extract of the highly infectious cottontail rabbit papillomas of W. R. 1-72 diluted 1:120 in saline as the antigen. The papilloma extracts and sera were also tested for capacity to neutralize a 5 per cent virus filtrate (W. R. 1-68) by rubbing the mixtures into scarified skin areas in three normal domestic rabbits according to the usual titration method.

The results of the tests are shown in Table V. It will be seen that the sera from D. R. 10 and 2-47 contained much antibody, as determined by both the complement fixation and virus neutralization tests, and extracts of the papillomas from these animals also fixed complement in high titer and almost completely neutralized the test virus. The serum of D. R. 2-47 had less antibody than that of D. R. 10 and the extract of the papillomas from this rabbit yielded slightly less inhibitor and fixed complement in lower titer than the extracts from D. R. 10. Furthermore, the sera fixed complement in higher titer than the papilloma extracts and, as previously noted (Experiment 2), had a greater neutralizing capacity. The sera of D. R. 24 and 2-50 had low antibody titers and extracts of the papillomas from these rabbits contained little or no inhibitor and did not react in the complement fixation test.

The experiment shows (Table V) that extracts of papillomas from rabbits with much blood antibody neutralize large amounts of the papilloma virus and also fix complement in high titer when mixed with the virus *in vitro*; whereas extracts of growths from rabbits with small amounts of blood

antibody yield little or no inhibitor and have no capacity to react in the complement fixation test. These findings afford further evidence of the identity of inhibitor and antibody, since, as already stated, the antibodies responsible for neutralization of the virus and for the fixation of complement in mixture with it are identical (3, 4). The highest dilution at which the papilloma extracts fixed complement was less than that of the serum of the host bearing the growths, as had been previously noted with the neutralization tests for inhibitor (see Table II).

Tests for Specific Absorption with the Inhibitor and the Virus, Respectively

The virus-neutralizing and complement-fixing antibodies are readily absorbed from the sera of rabbits bearing virus-induced papillomas, when these are mixed with extracts containing the papilloma virus (4). In view of this fact, tests were undertaken to see whether the inhibitor would be specifically absorbed from papilloma extracts when they were mixed with a filtrate containing the virus.

Experiment 7.—The papilloma extracts of rabbits D. R. 10 and 2-47, mentioned in the preceding experiment, were used as the sources of inhibitor. The technique of the absorption test was essentially that employed in the absorption of serum antibody (4). The papilloma extracts in dilution of 1:4 were mixed in equal parts with a highly infectious papilloma virus filtrate (W. R. 1-28) in dilutions of 1:10, 1:20, 1:40, and 1:80 in saline. The mixtures together with appropriate saline controls were incubated for 2 hours at 37°C. and left overnight in the refrigerator. The amount of visible flocculation in each tube was recorded and the mixtures were spun at about 4400 R.P.M. for 20 minutes. The amount of inhibitor remaining in the supernatant fluids was determined by complement fixation and virus neutralization tests.

Table VI shows that the inhibitor was completely removed from the papilloma extract of D. R. 10 when this was mixed with the virus filtrate in dilution of 1:10, as determined by both complement fixation and neutralization tests. Further dilution of the virus filtrate resulted in a corresponding reduction in the absorption of inhibitor. The papilloma extract of D. R. 2-47, which contained less inhibitor than that of D. R. 10, had none that was detectable after absorption with the virus filtrate in dilutions of 1:10 and 1:20, whereas partial absorption occurred with the filtrate diluted 1:40 and 1:80. A visible flocculation was present in those mixtures in which inhibitor was completely removed from the extracts, similar to that noted in optimal mixtures of immune serum and the papilloma virus (4). Subsidiary tests showed that no excess virus was present in any of the mixtures after absorption. The results illustrate incidentally the greater sensitiveness of the neutralization test for inhibitor, a finding which parallels the results of tests by both methods for serum antibody (3, 4). No inhibitor was detectable by the complement fixation test in the mixtures containing the papilloma extract D. R. 10 and virus filtrate 1:20, and extract D. R. 2-47 and virus filtrate 1:40, yet both caused slight neutralization of the test virus.

In the next experiment a constant amount of a virus filtrate was absorbed with papilloma extracts known to contain differing quantities of inhibitor.

Experiment 8.—Two papilloma extracts were utilized containing large amounts of inhibitor (D. R. 10 and 2-47) and two in which there was little or none (D. R. 24 and 2-50). Portions of them had been employed in Experiment 6. They were tested for

TABLE VI

Specific Absorption of Inhibitor with the Papilloma Virus

Source of inhibitor (papilloma extract 1:4)	Dilution of virus filtrate W R. 1-28 used for absorption	Inhibitor remaining in mixtures after absorption as determined by									
		Complement fixation tests†							Neutralization tests‡		
		Dilutions of extracts							Test rabbits		
		1:8	1:12	1:16	1:24	1:32	1:48	1:64	a	b	c
D. R. 10	1:10	0	0	0	0	0	0	0	****	****	***±
	1:20	0	0	0	0	0	0	0	**	***	*±
	1:40	++++	++++	++++	+	0	0	0	±	±	±
	1:80	++++	++++	++++	++++	+++±	+	0	±	0	0
	Unabsorbed; saline control....	++++	++++	++++	++++	++++	+++±	+	0	0	0
D. R. 2-47	1:10	0	0	0	0	0	0	0	****	****	***±
	1:20	0	0	0	0	0	0	0	****	****	***±
	1:40	0	0	0	0	0	0	0	***	***±	**
	1:80	+++±	±	0	0	0	0	0	*	*	±
	Unabsorbed; saline control....	++++	++++	++++	+++	±	0	0	±	±	0
5 per cent virus (1-68) plus saline....									***±	****	***±

† Complement, 2 units in all tubes.
Antigen, W. R. 1-72 extract, 1:120.
None of the materials was anticomplementary when tested in double amount.

‡ Growths resulting from mixture of 5 per cent virus filtrate (W. R. 1-68) and absorption mixture in three test rabbits on the 42nd day after inoculation.

capacity to absorb virus from a 5 per cent filtrate of the glycerolated cottontail rabbit papillomas of W. R. 1-28. The technique used in the preceding absorption experiment was followed.

It will be seen (Table VII) that the papilloma extracts containing a large amount of inhibitor (D. R. 10 and 2-47) completely absorbed the complement-fixing antigen and removed practically all of the infectivity of the virus suspension. (A single discrete growth occurred in one of the test rabbits.) By contrast the papilloma extract from rabbit D. R. 24, which contained a small amount of inhibitor, absorbed a slight amount of the complement-fixing antigen and the infectivity of the virus filtrate; and the extract of D. R. 2-50, which contained no inhibitor, absorbed no demonstrable amount of the antigen or the infectious virus.

The results of these tests (Tables VI and VII) clearly show that the inhibitor can be specifically absorbed from papilloma extracts with the virus and conversely, that the infectivity and the complement-fixing antigen of a virus filtrate can be absorbed with inhibitor. The findings are precisely like those previously reported for the absorption of serum antiviral antibody (4), and they provide further proof of the identity of inhibitor and antibody.

TABLE VII

Specific Absorption of the Papilloma Virus with Inhibitor

Source of inhibitor used for absorption of virus filtrate (W. R. 1-28)	Rabbit No.	Complement fixation tests† to determine amount of virus antigen remaining after the absorption					Pathogenicity tests‡ to determine amount of infectious virus remaining after the absorption					
		Dilutions of virus filtrate (W. R. 1-28)					17th day			42nd day		
		1:20	1:40	1:80	1:160	1:320	a	b	c	a	b	c
	D. R.											
Rabbits with high serum antibody titer, having papillomas which yielded much inhibitor	10	0	0	0	0	0	0	0	0	0	0	0
	2-47	0	0	0	0	0	0	0	0	0	±	0
Rabbits with low serum antibody titer, having papillomas which yielded little or no inhibitor	24	++++	++++	+++±	++	0	±	***±	*±	****	****	****
	2-50	++++	++++	++++	+++±	±	**	****±	***	****	****	****
Virus filtrate plus saline (control)		++++	++++	++++	+++±	±	**	****±	***±	****	****	****

† Complement, 2 units in all tubes.
Immune serum, D. R. 1-65, 1:24.
None of the materials was anticomplementary when tested in double amount.

‡ Growths in test rabbits (a, b, c) according to standard scale.

Effect of Heat on Inhibitor and Antiviral Antibody

In extension of the observations comparative tests were now made of the effect of heat on the inhibitor and serum antiviral antibody.

Experiment 9.—The effect of heat on three immune sera was tested by means of complement fixation tests. The sera were obtained from rabbits carrying papillomas of about 16 weeks' duration (D. R. 1-36, 9-52, 1-53), which had received two intraperitoneal injections of a virus filtrate (W. R. 1-70) to stimulate a high antibody titer. The sera were diluted 1:10 in saline and 3 cc. portions put into sealed glass tubes and submerged in water baths at temperatures varying from 60° to 85°C. for 30 minutes. The sera heated at 60° and 65° showed no visible change, while those heated at 70° and 75°

had a faint bluish opalescence, and this was more pronounced in the tubes heated at 80° and 85°. No gross flocculation was present in any of the heated sera, however. The unheated and heated sera were tested by means of complement fixation tests in the usual way.

The serum antiviral antibody proved to be fairly resistant to heat (Table VIII). In every case heating at 80°C. for 30 minutes inactivated it completely, and there was no detectable antibody in two of the sera after heating at 75°C. Heating at 70°C. inactivated it partially, whereas temperatures of 60° and 65° had no detectable effect. The destruction of the antibody was attended by an increasing opalescence of the sera.

TABLE VIII

The Effect of Heat on the Blood Antibody

Serum	Complement fixation tests†											
Heated 30 min.	Serum D. R. 1-53				Serum D. R. 1-36				Serum D. R. 9-52			
	Dilutions of serum				Dilutions of serum				Dilutions of serum			
	1:10	1:20	1:40	1:80	1:10	1:20	1:40	1:80	1:10	1:20	1:40	1:80
°*C.*												
Unheated	++++	++++	++++	+++±	++++	++++	+++±	++	++++	++++	+++±	±
60	++++	++++	++++	+++±	++++	++++	+++±	++	++++	++++	+++±	±
65	++++	++++	++++	+++±	++++	++++	+++±	++	++++	++++	+++±	±
70	++++	++++	+++±	+±	++++	+++±	0	0	++++	+++±	±	0
75	++	0	0	0	0	0	0	0	0	0	0	0
80	0	0	0	0	0	0	0	0	0	0	0	0
85	0	0	0	0	0	0	0	0	0	0	0	0

† Complement, 2 units in all tubes.
Antigen, virus extract W. R. 1-70, 1:120.
None of the materials was anticomplementary when tested in double amount.

The experiment shows that the serum antiviral antibody has a considerable resistance to heat. Is this true of the inhibitor in papilloma extracts as well? Comparative tests on the point were undertaken.

Experiment 10.—The papilloma extracts and sera of two of the rabbits of the preceding test (D. R. 1-36 and 9-52) were used in this experiment. 3 cc. portions of each, diluted 1:10 in saline, were heated in water baths at temperatures of 60° to 85°C. for 30 minutes. An abundant flocculent precipitate formed in the heated papilloma extracts. To learn whether it contained the inhibitor, one of the extracts (D. R. 9-52) was spun at 3500 R.P.M. for 10 minutes, and the supernatant fluids were removed and the sediments resuspended in the original volume of saline. Both were tested for inhibitor, as were also whole heated specimens of the extract of D. R. 1-36. None of the sera showed any precipitate after heating, but samples heated above 70° were opalescent, as noted in the preceding experiment. The unheated and heated sera and extracts were tested for capacity to neutralize a 1 per cent virus filtrate (W. R. 77).

Table IX shows the results of these tests. The inhibitor and serum antibody were completely inactivated by heating at 80° and 85°C. for 30 minutes, while 75° caused

partial destruction of both. 70°C. caused partial destruction of the inhibitor in the papilloma extract of D. R. 9-52, but there was no detectable decrease in the neutralizing capacity of the extract of D. R. 1-36 when heated at these temperatures. This latter extract, however, contained a larger amount of inhibitor. 60° and 65°C. had no detectable effect on either inhibitor or antibody. The precipitate removed from the heated extracts of D. R. 9-52 by centrifugation contained no detectable inhibitor. This finding has not been included in the table.

The inhibitor in papilloma extracts and the serum neutralizing antibody showed an essentially identical resistance to heat. Exposure to 80°C. for

TABLE IX

The Effect of Heat on Inhibitor and Blood Antibody

Material heated 30 min.	Neutralization tests†											
	Virus filtrate plus											
	Papilloma extract (D. R. 1-36)			Serum (D. R. 1-36)			Papilloma extract (D. R. 9-52)			Serum (D. R. 9-52)		
	a	b	c	a	b		d		f	d	e	f
°C.												
Unheated	0	0	0	0	0	0	0	0	0	0	0	0
60	0	0	0	0	0	0	0	0	0	0	±	0
65	±	0	0	0	0	0	0	±	0	±	0	0
70	0	0	0	0	0	0	*	*	±	±	0	0
75	±	±	*	±	±	±	**	**	**	±	±	±
80	****	****	***±	***±	****	****	****	***	****	****	****	****
85	****	****	****	****	****	****	****	****	****	****	****	****
Virus filtrate plus saline..........	****	****	****	****	****	****	****	***±	***±	****	****	***±

† Virus filtrate W. R. 77-E, 1:100.
Papilloma extracts and sera diluted 1:10 in saline.
Growth in test rabbits (a, b, c, d, e, f) on the 35th day after inoculation.

30 minutes proved necessary for their total destruction, whereas 60° and 65°C. were without effect on either. These findings extend those on the destruction of antibody as determined by complement fixation tests (Table VIII).

Precipitation of Inhibitor and Antibody with Ammonium Sulfate

Antibodies can usually be precipitated with the globulin fraction of sera by a variety of methods (7). Tests were now made to see whether the inhibitor and serum antiviral antibody are precipitated by the same concentrations of ammonium sulfate.

Experiment 11.—Portions of the sera and papilloma extracts obtained from rabbits D. R. 1-36 and 9-52 for the purposes of the preceding test were utilized, as were those

from a third rabbit, D. R. 1-35, which had also received intraperitoneal injections of a virus filtrate to stimulate antibody production. After each had been diluted 1:10 wi h saline, it was mixed with a solution of ammonium sulfate to a final concentration of ⅓ saturation in order to precipitate the *euglobulin fraction.* The mixtures were kept in a refrigerator (4°C.) for 5 hours. A heavy precipitate formed in the bottom of each tube and the visible amount in each was approximately the same. They were then spun at 4400 R.P.M. for 20 minutes, the supernatant fluids were removed, and the sediments resuspended in the original volume of saline (4 cc.). The resuspended serum euglobulin solutions appeared water-clear, whereas those of the papilloma extracts were slightly turbid and a few small particles could be seen. Now more ammonium sulfate,

TABLE X

Precipitation of Inhibitor and Blood Antibody with $(NH_4)_2SO_4$

Material tested		Neutralization tests: Virus filtrate† plus material from rabbits								
		D. R. 1-36			D. R. 9-52			D. R. 1-35		
		a	b	c	a	b	c	d	e	f
Papilloma extract (1:10)	Whole extract	0	0	0	0	0	0	0	0	0
	Euglobulin fraction‡	0	0	0	±	+	+	+	±	±
	Pseudoglobulin " §	0	0	0	±	0	±	±	±	±
Serum (1:10)	Whole serum	0	0	±	0	0	0	0	0	0
	Euglobulin fraction	0	0	±	0	0	±	0	0	±
	Pseudoglobulin "	0	0	0	0	±	0	±	0	0
Virus filtrate plus saline		+++±	+++±	++++	+++±	+++±	++++	++++	+++±	++++

† Virus filtrate W. R. 1-68, 1:100.
Growths in test rabbits (a, b, c, d, e, f) on 35th day after inoculation.
‡ Precipitated with ⅓ saturation ammonium sulfate.
§ Precipitated with ½ saturation ammonium sulfate.

to the extent of ½ saturation, was added to precipitate the *pseudoglobulin fraction.* The mixtures were kept overnight in the refrigerator and all had a heavy, large-flocculent precipitate. They were spun at 4400 R.P.M. for 20 minutes, the supernatant fluids removed, and the sediments resuspended in the original volume of saline. The resuspended pseudoglobulin solutions were all water-clear.

The original papilloma extracts and sera and the euglobulin and pseudoglobulin fractions of each were tested for capacity to neutralize a 1 per cent virus filtrate (W. R. 1-68 E). The extracts and sera that had been precipitated with ½ saturated ammonium sulfate were also tested for neutralizing capacity in dilution of 1:20, as were the original extracts and sera similarly diluted.

Table X shows that the globulin fractions salted out of "inhibiting" extracts and neutralizing sera had nearly as great an ability to neutralize virus as the whole materials. It is evident that inhibitor and antibody

are alike in their precipitation reactions with ammonium sulfate, and the results at ⅓ and ½ saturation indicate that they are distributed between the euglobulin and pseudoglobulin fractions. A small amount of inhibitor and antibody remained in the extracts and sera, respectively, following the precipitation with ½ saturated ammonium sulfate (not shown in the table). Subsidiary tests showed that the inhibitor and serum antibody could be also precipitated with absolute alcohol and with acetone.

Effect of Perfusion on Yield of Inhibitor and Virus from Papillomas

Tests were now undertaken to see whether the inhibitor could be removed from the papillomas by perfusing the rabbit host with saline immediately after it had been killed.

Experiment 12.—A domestic rabbit (D. R. 1-56) and a cottontail rabbit (W. R. 23) each had a large confluent and six small discrete papillomas. Each received two intraperitoneal injections of a virus filtrate at weekly intervals to raise the circulating antibody to a high titer. The rabbits were then bled from the heart for serum and killed. A portion of the large papillomatous mass of each rabbit was immediately isolated by means of a long curved clamp placed across the base and through a portion of the growth. Thus while a portion of the papilloma remained intact the remainder (clamped portion) was isolated from the general circulation. Clamps were also placed at the bases of three of the small discrete papillomas of each rabbit, while the vessels of the remaining three were not blocked. A portion of abdominal skin was similarly isolated. The heart was then cut across through the lower half of the two ventricles, a glass cannula was inserted into the aorta through the left ventricle, and about 3 liters of warm saline (0.9 per cent) was run in from a flask 3 feet above the heart. The blood vessels that had been cut while exposing the heart had been tied to prevent leakage and the saline returned through the right ventricle after passing through the rabbit. Using this same technique in other rabbits, it was found that an India ink-gelatin solution would fill the blood vessels throughout the living portions of the papillomas. The perfused and non-perfused papillomas and skin were removed from each animal with different sets of sterile instruments and 10 per cent saline extracts of them were prepared. The extracts and sera, all in dilution of 1:10, were tested for capacity to neutralize a 1 per cent virus filtrate (W. R. 1-30). The extracts were also tested for infectiousness by means of pathogenicity tests.

The tests disclosed no very great difference in the yield of inhibitor from the perfused and non-perfused papillomas or skin of the domestic or cottontail rabbits (Table XI). Nevertheless the cottontail papillomas that were perfused had a greater infectivity on extraction, notably the small discrete growths, which contained less inhibitor than did the large confluent papillomas. Hence it seems a fair inference that some fraction of the inhibitor had been removed. Most of it, however, doubtless lay outside the vessels as result of extravasation, and hence not likely to be removed when they were flushed.

Relation of Inhibitor to the Recovery of Virus from the Papillomas of Domestic Rabbits

The papilloma virus cannot be recovered ordinarily from the growths induced with it in domestic rabbits; yet the virus is known to be present in them, for the titer of circulating antibody increases as the papillomas enlarge (2). Kidd has shown that little or no virus can be procured from

TABLE XI

Effect of Perfusion on the Yield of Inhibitor and of Virus from Papillomas

Material		Tests for inhibitor†						Pathogenicity tests			
		Growths resulting from the mixture of virus filtrate and extracts from						Extracts from cottontail rabbit (W. R. 23)			
		Domestic rabbit (D. R. 1-56)			Cottontail rabbit (W. R. 23)			17th day after inoculation		35th day after inoculation	
		a	b	c	d	e	f	g	h	g	h
Papilloma extracts from (1) Small discrete growths	Not perfused	0	0	0	***	*±	**	0	0	*	*
	Perfused	0	0	0	***	**	***	*±	*±	***±	****
(2) Large confluent growths	Not perfused	0	0	0	*±	*	±	0	0	0	0
	Perfused	0	0	±	*	**	**	0	0	±	±
Skin extract	Not perfused	±	±	±	*	*	±				
	Perfused	±	*	*	*	*±	±				
Serum		0	0	0		0	0				
Controls (virus filtrate plus saline)		***±	***±	****	****	****	****				

† 1 per cent virus filtrate (W. R. 1-28) and 10 per cent extracts in equal parts.
Readings in test rabbits (a–h) on 35th day after inoculation according to the standard scale·

the growths of domestic rabbits, even when only small amounts of antibody are present in the sera of the hosts (6). He concluded therefore that something other than antibody is primarily responsible for the "masking" of the virus in this species. The question arises as to whether the inhibitor has anything to do with the failure to recover virus from extracts of these papillomas. A number of observations made during the course of this work bear upon the problem.

A total of 63 papillomas from 36 rabbits were tested for virus and inhibitor during the course of the experiments reported in this paper. Fifty-one of these papillomas were obtained from 30 rabbits with considerable amounts of antibody in their blood. Extracts

of the papillomas all contained inhibitor and none of them yielded virus upon inoculation into the scarified skin of test rabbits. Twelve papillomas were obtained from 6 rabbits with little or no blood antibody and the results of the tests with this group revealed a striking correlation between the yield of inhibitor and virus from them. The discrete and confluent papillomas resulting from tattoo inoculation and inunction, respectively, of a single virus material into the rabbits of Experiment 2 were tested concurrently for yield of virus and inhibitor. The growths had been washed with soap and thoroughly rinsed with water before removal from the hosts by operation, in order to remove any blood or virus that might be present on their surface. Afterwards they were diced into small pieces and passed through several changes of saline, again for the purpose of removing any adherent blood or virus. Portions of the extracts of the discrete and confluent papillomas, which were obtained on the 21st, 37th, and 111th days after virus inoculation and prepared as described in Experiment 2, were tested for virus by rubbing them into scarified skin areas of normal domestic rabbits. (The confluent growths were large and provided an abundance of material. They were extracted 1:10 in saline. The discrete growths, however, were small and hence had to be extracted 1:20.) The extracts were also tested for inhibitor in the usual way, but with a 1 per cent suspension of the W. R. 1-10 filtrate as the test virus instead of 5 per cent, as used in Experiment 2, in order to render the test somewhat more sensitive. The blood antibody titer of the rabbits was determined by the neutralization and complement fixation tests.

The results of the tests with the extracts and sera obtained on the 21st day after inoculation are summarized in Table XII. (The serum neutralization tests are not included in the table, since they did not differ from those recorded in Table II. One of the three test rabbits used in the pathogenicity tests and one of the group used in the inhibitor tests died soon after inoculation, and hence the results in only two rabbits of each group are available.) It will be seen that the papillomas from D. R. 100, which had no detectable blood antibody, contained no inhibitor and that they yielded a small amount of virus, extracts of the growths producing from 4 to 22 papillomas when inoculated into the test rabbits. Extracts of the papillomas from D. R. 98, 99, and 101 caused just perceptible neutralization of the test virus and the blood antibody titer of these rabbits was quite low. Some of these papillomas yielded virus, namely, the discrete growths from D. R. 98 and 99 and the confluent growths from D. R. 98 and 101, but the amount was less than that from the papillomas of D. R. 100. The blood of D. R. 96 and 97 contained antibody in higher titer than the other four rabbits and extracts of the papillomas contained considerable amounts of inhibitor. These growths yielded no virus.

The results of similar tests with portions of the papillomas of four of the rabbits (D. R. 96, 97, 100, and 101) obtained on the 37th and 111th days can be briefly summarized. (D. R. 98 had died, and the growths of D. R. 99 had retrogressed, so that their papillomas were not available for test.) The serum antibody titers of these rabbits had risen since the 21st day and inhibitor could now be detected in extracts of all of the growths in increased amount (Table II). No virus was obtained from any of the papillomas in these later tests.

The findings indicate that virus cannot be recovered ordinarily from the papillomas of domestic rabbits if they contain any considerable amount of inhibitor. Papillomas obtained from rabbits early in their development,

TABLE XII

Yield of Virus and Inhibitor from the Papillomas of Domestic Rabbits

Rabbit No.	Character of the growths	Test rabbits	Pathogenicity tests with extracts† of the growths			Test rabbits	Tests for inhibitor 1 per cent virus filtrate‡ plus		Serum antibody titer: Complement fixation tests: Dilutions of serum			
			18th day	28th day	42nd day		Saline (controls)	Papilloma extracts	1:2	1:4	1:8	1:12
100	Discrete	a	±	±	±	c	***±	***±	0	0	0	0
		b	±	±	±	d	****	****				
	Semiconfluent	a	*	**	**	c	***±	***±				
		b	0	0	0	d	****	****				
98	Discrete	a	0	±	±	e	***±	***±	+++±	0	0	0
						f	***±	**±				
		b	0	0	0	g	***	***				
	Semiconfluent	a	0	±	±	e	***±	***				
						f	***±	***				
		b	0	±	±	g	***	**±				
99	Discrete	a	±	*	*	c	***±	***	+++	0	0	0
		b	0	0	0	d	****	***				
	Semiconfluent	a	0	0	0	c	***±	***				
		b	0	0	0	d	****	***				
101	Discrete	a	0	0	0	c	***±	***	+++	±	0	0
		b	0	0	0	d	****	***				
	Semiconfluent	a	0	0	0	c	***±	***				
		b	0	±	±	d	****	***				
97	Discrete	a	0	0	0	e	***±	*±	++++	++++	±	0
						f	****	**				
		b	0	0	0	g	***	*±				
	Semiconfluent	a	0	0	0	e	***±	±				
						f	***±	**				
		b	0	0	0	g	***	±				
96	Discrete	a	0	0	0	e	***±	*	++++	++++	+++±	±
						f	****	*±				
		b	0	0	0	g	***	**				
	Semiconfluent	a	0	0	0	e	***±	**				
						f	****	**				
		b	0	0	0	g	***	*±				

† Discrete papillomas extracted 1:20 in saline.
Semiconfluent papillomas extracted 1:10 in saline.

‡ Virus filtrate W. R. 1-10.
Growths on 35th day after inoculation.

when the blood contains little or no antibody and extracts of the growths contain little or no inhibitor, may yield small amounts of the virus. The amount of virus recovered from the papillomas was never large. 5 to 10 per cent saline extracts of the growths produced from one to 22 discrete papillomas when inoculated into the scarified skin of test rabbits, and the incubation period was prolonged, varying from 18 to 30 days. It is possible that the small amount of virus that was finally recovered had merely persisted on or about the growths from the original inoculum, but the fact that the papillomas were thoroughly washed before extraction makes this possibility seem unlikely.

In contrast to these findings, it is not unusual for the papillomas of cottontail rabbits to yield extracts capable of producing growths after dilution to 1:200,000. Manifestly the inhibitor, like the antiviral antibody, cannot account for the failure to recover virus from the papillomas of domestic rabbits in amount comparable to that procured from wild rabbit growths, for even in its absence only a comparatively small amount of virus could be recovered, as Kidd has also found (6). It appears, however, that the inhibitor when present in considerable amount renders it impossible to recover virus from the papillomas of domestic rabbits with the methods now in use.

DISCUSSION

The prime purpose of the experiments here reported has been to learn whether the virus inhibitor, which is often present in extracts of the virus-induced rabbit papillomas, is identical with antiviral antibody derived from the blood. The evidence may now be briefly recapitulated. There was found to be a quantitative relationship between the inhibitor in papilloma extracts and the serum antiviral antibody. Papillomas tested before the specific antibody could be detected in the serum of the host in any significant amount yielded none of the inhibitor, nor was it present in extracts of normal rabbit tissues or the Brown-Pearce tumor. The inhibitor was not confined to the papillomas but was present in extracts of liver, muscle, and skin from rabbits bearing the papillomas,—and in amounts proportional to the titer of serum antibody. The inhibitor, like the antiviral antibody (2), had no discernible influence on the course of the papillomas, these enlarging progressively or dwindling and vanishing irrespective of their content of inhibitor. Both were completely inactivated by heating at 80°C. for 30 minutes, and they were precipitated together upon treatment with ammonium sulfate. The inhibitor fixed complement in mixture with the papilloma virus in proportion to its neutralizing capacity and it was specifi-

cally absorbed from papilloma extracts when mixed with the papilloma virus, and the infectivity and complement-fixing antigen of a virus filtrate could be completely absorbed with the inhibitor,—findings similar to those previously reported for the blood antibody (3, 4). Taken together these facts prove the inhibitor to be identical with serum antibody.

The inhibitor or antibody—for the terms can be used interchangeably—is readily studied in extracts of the papillomas of domestic rabbits, because the virus, though responsible for the growths and persisting in them (2), is not present in any considerable amount in active form and hence does not act to absorb the inhibitor. The papillomas of cottontail rabbits, on the other hand, usually yield large amounts of the virus, and it would follow that any inhibitor present is doubtless absorbed by virus and hence cannot be demonstrated. In such instances one must suppose the virus to be present in excess, over and above the amount combining with the inhibitor in the extract. Not infrequently, however, so much inhibitor is present, under conditions favorable to the extravasation of antibody in large amount, that the virus is wholly "masked" (6). It is in such instances that inhibitor can be detected in extracts of the cottontail growths, again as representing an excess.

The amount of inhibitor in extracts of freshly procured papillomas from domestic rabbits with high titers of serum antibody is considerable, 0.2 cc. of a 5 per cent suspension neutralizing in some instances an amount of virus equivalent to 2000 or more minimal infectious doses. As already remarked, the amount of detectable antibody (inhibitor) in papilloma extracts was always less than that present in the blood of the host, the titer of the former being roughly one-fourth to one-eighth that of the serum (Tables II and V). Freund (8) found a rather constant numerical relationship between the antibody content of the blood and organs following the intravenous injection of rabbit serum containing typhoid agglutinins into rabbits, the amount in the organs being on the average about one-tenth that in the serum. The greater proportion of antibody in papilloma extracts is probably accounted for by the greater opportunity afforded for extravasation and localization of antibody in the papillomas than in normal tissues. It seems probable, as has been stated, that most of the inhibitor present in extracts of the papillomas represents antibody that has become localized in the tissues, not merely circulating antibody. This is suggested by the finding that extracts of organs containing large amounts of blood (liver) contain no more inhibitor than do extracts of the papillomas (Experiments 3 and 4), and furthermore by the failure of perfusion with saline to effect any large reduction in the inhibitor content of the papillomas (Experiment 12).

The results of Freund's experiments with typhoid agglutinins in normal tissues are quite similar (8).

Certain virus inhibitors have been encountered in other diseases, which may now be considered in relation to the findings with the inhibitor in the rabbit papillomas. Extracts of slowly growing chicken tumors often contain an inhibitor that neutralizes the infectious tumor agent *in vitro* (9, 10, 11). Some of the properties of this inhibitory substance suggest that it is neutralizing antibody of the sort found in the blood. Andrewes has found that the blood of chickens bearing tumors of slow growth usually contains considerable amounts of neutralizing antibody (12). Furthermore, the inhibitor present in extracts of the chicken tumors is a globulin (10, 11), and like the neutralizing antibody for the chicken tumor virus it forms an unstable union in mixture with the latter, which can be readily dissociated by centrifugation or by adsorption on aluminum hydroxide (10, 12). Nasal secretions from human subjects often contain a substance capable of inactivating relatively large amounts of influenza virus (13, 14). Francis found a relationship between the neutralizing effect of the sera and that of the nasal secretions, particularly in patients with blood antibody titers of 1:40 or more. He also noted that the neutralizing antibodies and the agent in the nasal secretions were inactivated at the same temperature (14). The findings as a whole support the view that the specific virus inhibitors of the chicken tumors and of the human nasal secretions may be, like the inhibitor of the papilloma virus, extravasated antiviral antibody. Papilloma virus inhibitor has also been demonstrated in extracts of the cancers deriving from the virus-induced growths of cottontail and domestic rabbits (15), and it is apparently identical with the inhibitor found in the papillomas.

SUMMARY

The "inhibitor" demonstrable in extracts of the virus-induced rabbit papillomas is identical with the antiviral antibody found in the blood of hosts bearing the growths. The conditions in these latter are frequently favorable to its extravasation in considerable amount into them. Its significance and its influence upon the recovery of virus from the papillomas are discussed.

BIBLIOGRAPHY

1. Shope, R. E., *J. Exp. Med.*, 1933, **58**, 607.
2. Kidd, J. G., Beard, J. W., and Rous, P., *J. Exp. Med.*, 1936, **64**, 63, 79.
3. Kidd, J. G., *J. Exp. Med.*, 1938, **68**, 703, 725, 737.
4. Friedewald, W. F., and Kidd, J. G., *Proc. Soc. Exp. Biol. and Med.*, 1939, **42**, 330.

5. Kidd, J. G., *J. Exp. Med.*, 1940, **71**, 335, 351.
6. Kidd, J. G., *J. Exp. Med.*, 1939, **70**, 583.
7. Marrack, J. R., The chemistry of antigens and antibodies, *Great Britain Med Research Council, Special Rep. Series, No. 230*, 1938.
8. Freund, J., *J. Immunol.*, 1927, **14**, 101.
9. Sittenfield, M. J., Johnson, B. A., and Jobling, J. W., *Proc. Soc. Exp. Biol. and Med.*, 1931, **28**, 517.
10. Murphy, Jas. B., Helmer, O. M., Claude, A., and Sturm, E., *Science*, 1931, **73**, 266. Murphy, Jas. B., and Sturm, E., *J. Exp. Med.*, 1932, **56**, 107.
11. Claude, A., *Am. J. Cancer*, 1939, **37**, 59.
12. Andrewes, C. H., *J. Path. and Bact.*, 1931, **34**, 91; 1932, **35**, 243.
13. Burnet, F. M., Lush, D., and Jackson, A. V., *Brit. J. Exp. Path.*, 1939, **20**, 377.
14. Francis, T., Jr., *Science*, 1940, **91**, 198.
15. Kidd, J. G., and Rous, P., *J. Exp. Med.*, 1940, **71**, 469.

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EFFECTS OF HEXYLRESORCINOL ON NITELLA

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The effects of hexylresorcinol[1] show some resemblance to those of guaiacol,[2, 3] as might be expected in view of their chemical similarity.

But interesting differences exist. Guaiacol increases the mobility of Na^+ (u_{Na}) and leaves that of K^+ (u_K) unchanged, but hexylresorcinol decreases[4] both mobilities. Hexylresorcinol 0.0003 M brings about as much negative change in P.D. as does 0.03 M guaiacol. The latter is not toxic in brief exposures (up to 5 minutes) but with the same exposure hexylresorcinol shows some toxicity at 0.003 M since some cells recover their normal P.D. when replaced in 0.001 M NaCl but others fail to do so. At 0.0003 M complete recovery may occur in a few minutes but such cells do not always live well afterwards.

Fig. 1 shows the effect of applying[5] 0.0003 M hexylresorcinol.[6] The curve

[1] $C_6H_3(OH)_2(C_6H_{13})$.

[2] $C_6H_4(OH)(OCH_3)$ 1:2.

[3] Osterhout, W. J. V., *J. Gen. Physiol.*, 1938-39, **22**, 417; 1939-40, **23**, 171.

[4] It is assumed that the mobility of Cl^- (v_{Cl}) remains unchanged since v_{Cl} is taken as unity. *Cf.* Osterhout, W. J. V., *J. Gen. Physiol.*, 1929-30, **13**, 715.

[5] The cells, after being freed from neighboring cells, stood in the laboratory at 15° ± 1°C. in Solution A (*cf.* Osterhout, W. J. V., and Hill, S. E., *J. Gen. Physiol.*, 1933-34, **17**, 87) until used. They belonged to Lot B (*cf.* Hill, S. E., and Osterhout, W. J. V., *Proc. Nat. Acad. Sc.*, 1938, **24**, 315) unless otherwise stated.

The measurements were made on *Nitella flexilis*, Ag., using the technique described in former papers (Hill, S. E., and Osterhout, W. J. V., *J. Gen. Physiol.*, 1937-38, **21**, 541). The temperature varied from 20 to 29°C.

Unless otherwise stated there was no indication of injury.

Two spots, *D* and *E*, were recorded but the record of *E* is omitted in the reproductions given here. Both *D* and *E* were connected through the galvanometer to a spot *G* at the end of the cell. Any change at *G* would be revealed by simultaneous changes in the records of *D* and *E*: no such change occurred in the records here given.

After an exposure of some minutes with 0.0003 M hexylresorcinol at *D* and 0.01 M NaCl at *E* there was sometimes a sudden loss of P.D. at *E*, as though hexylresorcinol had passed from *D* to *E*, a distance of 1 cm. where the cell was surrounded by moist air: since hexylresorcinol is surface-active this may have played a rôle.

[6] The hexylresorcinol was kindly donated by the firm of Sharp and Dohme of Glenolden, Pa.

records the difference between two spots, *D* (in contact with 0.01 M NaCl) and *G* (in contact with 0.01 M KCl which reduces the P.D. approximately to zero). At the start, *D* had a positive[7] P.D. of about 65 mv. When the reagent was applied at *D*, the curve, after a latent period of about 32 seconds, rose slowly, indicating a loss of P.D. The latent period and the slow rise of the curve recall the effects of guaiacol on *Nitella*,[8] *Halicystis*,[9] and *Valonia*.[10]

The average duration of the latent period was about 25 seconds with 0.0003 M hexylresorcinol: it became shorter as the concentration was increased.[11] It seems possible that this is due, in part at least, to the time necessary for the reagent to penetrate through the protoplasm to the inner protoplasmic surface, *Y*, which is the chief seat[12] of the P.D.

When the depression of the P.D. reaches a certain point it may call forth an action current,[13] as seen in Fig. 2. This has also been observed when the P.D. is depressed[14] by KCl and has been explained as due to the discharge from a neighboring region.

A few cells gave curves like that shown in Fig. 3. Here the record shows the difference in P.D. between two spots, *D* and *G*, both in contact with 0.01 M NaCl. On applying hexylresorcinol at *D* the curve fell and then rose, indicating an increase in the positive[15] P.D. followed by a decrease. The promptness of the initial change indicates that the effect is on the outer surface of the protoplasm. The subsequent rise of the curve appears to be of the usual sort, probably involving penetration to the inner protoplasmic surface.

[7] The P.D. is called positive when positive current tends to flow from the vacuole across the protoplasm to the external solution.

[8] Osterhout, W. J. V., *J. Gen. Physiol.*, 1938–39, **22**, 417.

[9] Osterhout, W. J. V., *J. Gen. Physiol.*, 1937–38, **21**, 707.

[10] Osterhout, W. J. V., *J. Gen. Physiol.*, 1936–37, **20**, 13. In this case the P.D. becomes more positive.

[11] The values varied from 3 to 180 seconds.

[12] *Cf.* Osterhout, W. J. V., *J. Gen. Physiol.*, 1934–35, **18**, 215. The upward movement of the curve also occurs when we apply 0.0003 M hexylresorcinol dissolved in distilled water to a spot previously in contact with distilled water. Hence it does not depend on the salts in contact with the external surface.

[13] This may be propagated along the cell but usually is not. It is more apt to be propagated when the latent period is short.

[14] Hill, S. E., and Osterhout, W. J. V., *J. Gen. Physiol.*, 1937–38, **21**, 541.

[15] A change in the positive direction is produced by guaiacol in *Valonia*. Osterhout, W. J. V., *J. Gen. Physiol.*, 1936–37, **20**, 13.

In order to get at the cause of the positive change determinations were made of the concentration effects of NaCl and KCl. With normal cells

FIG. 1. Shows the effect of hexylresorcinol 0.0003 M. At the start the recorded spot (*D*) in contact with 0.01 M NaCl had an outwardly directed (positive) P.D. of about 65 mv. When its contact with the solution was broken the curve jumped to *F*, the free grid of the amplifier. Contact was then made (marked by arrow) with 0.01 M NaCl + 0.0003 M hexylresorcinol and after a latent period of about 32 seconds the curve slowly rose approximately to zero. (The zero is labelled "App. zero" since it is only an approximation.)

Two spots, *D* and *E* (1 cm. apart), were connected through the galvanometer to a spot *G* at the end of the cell in contact with 0.01 M KCl (which reduced the P.D. at *G* approximately to zero). Any change at *G* would be shown by simultaneous changes in the records of *D* and *E*. No such change occurred. The record of *E* (not shown here) shows that no changes occurred at *E*.

Vertical marks 15 seconds apart.

The cell was freed from neighboring cells and kept 5 days in Solution A at 15 ±1°C. The experiment was performed at 21°C.

FIG. 2. Shows an action current induced by hexylresorcinol. At the start the spot recorded (*D*) in contact with 0.015 M NaCl had a positive P.D. of about 95 mv. When its connection with the solution was broken the curve jumped to *F*, the free grid of the amplifier. It was then placed in contact with 0.015 M NaCl + 0.0003 M hexylresorcinol. After a latent period of about 27 seconds the curve rose gradually until an action current occurred.

Two spots, *D* and *E*, were recorded (the record of *E* is not shown): both were connected through the galvanometer to a spot *G* at the end of the cell in contact with 0.01 M KCl (which reduces the P.D. approximately to zero). The records show that there was no change of P.D. at this spot or at *E* during the experiment.

Vertical marks 15 seconds apart.

The cell was freed from neighboring cells and kept for 3 days at 15 ± 1°C. in Solution A. The experiment was performed at 23°C.

the following values were obtained. The average concentration effect[16] of NaCl (0.01 M followed by 0.001 M or *vice versa*) varied from 20 to 41 mv.

[16] The dilute solution is positive in the external circuit.

(depending on which lot of cells was measured). The corresponding values for KCl[17] are 28 to 49 mv.

During exposure to hexylresorcinol 0.0003 M the concentration effect[18] of NaCl falls off and may approach zero.[19] This indicates that the mobility of Na^+ (u_{Na}) is approaching that of Cl^- (v_{Cl}).

When this happens, NaCl has less tendency to lower the P.D. and the result is a downward (positive) movement of the curve, as seen in Fig. 3. But if the change in u_{Na} is delayed this effect may be masked by the tendency of the curve to rise as the result of other changes. This happens in

FIG. 3. Shows an effect of 0.003 M hexylresorcinol. At the start the spot recorded, *D*, in contact with 0.01 M NaCl, had an outwardly directed (positive) P.D. of about 103 mv. When its contact with the solution was broken the curve jumped to *F*, the free grid of the amplifier. It was then placed in contact with 0.01 M NaCl + 0.003 M hexylresorcinol. The curve fell, indicating an increase in P.D. and then rose approximately to zero which is here taken as though 0.01 M KCl were at *G*.

Two spots, *D* and *E*, were connected through the galvanometer to a spot *G* at the end of the cell in contact with 0.01 M NaCl. The record of *E* (not shown here) shows that no change occurred at *E* or *G* during the experiment.

Vertical marks 5 seconds apart.

The cell was freed from neighboring cells and kept for 5 days in Solution A at 15 ± 1°C. The experiment was performed at 29°C.

many cases, as seen in Figs. 1 and 2. The duration of the positive dip therefore varies considerably.

The concentration effect of KCl also falls off during exposure to hexylresorcinol indicating that u_K is approaching v_{Cl}.

Before exposure the average potassium effect, *i.e.* 0.01 M KCl followed by 0.01 M NaCl, amounts to from 53 to 76 mv. (depending on which lot

[17] When 0.001 M KCl is followed by 0.01 M, action currents may occur which make the change in P.D. unduly large. *Cf.* Hill, S. E., and Osterhout, W. J. V., *J. Gen. Physiol.*, 1937–38, **21,** 541. In all cases the change was made from 0.01 to 0.001 M.

[18] *I.e.*, the effect of substituting 0.001 M for 0.01 M, both solutions containing 0.0003 M hexylresorcinol.

[19] Although the test may be made after the curve has risen approximately to zero the spot is not dead for it recovers its normal P.D. when the hexylresorcinol is removed unless the exposure has been prolonged beyond 5 minutes and even then there is recovery in many cases.

of cells is measured). During exposure to the reagent this also falls off. This indicates that K^+ and Na^+ are becoming more alike in respect to mobility[20] or partition coefficient or both.

It may be added that the variability of the cells used in these experiments was unusually great. This was due in part to the fact that they were collected at intervals throughout the year and covered an unusual range of seasonal variations.

A recent paper by Höber and coworkers[21] states that hexylresorcinol reversibly depresses the resting P.D. of frog muscle and of frog nerve. They ascribe this to a dispersing effect on the colloids of the surface. It is probable that hexylresorcinol produces structural changes in *Nitella* but the nature of these alterations requires further investigation.

SUMMARY

In some ways the effects of hexylresorcinol on *Nitella* resemble those of guaiacol but in others they differ.

Both substances depress the P.D. reversibly and both decrease the potassium effect.

Hexylresorcinol decreases the apparent mobility of Na^+ and of K^+. Guaiacol increases that of Na^+ but not of K^+.

The action of hexylresorcinol is more striking than that of guaiacol since 0.0003 M of the former is as effective as 0.03 M of the latter in depressing the P.D.

It is evident that organic substances can change the behavior of inorganic ions in a variety of ways.

[20] Regarding this see footnote 3.

[21] Höber, R., Andersh, M., Höber, J., and Nebel, B., *J. Cell. and Comp. Physiol.*, 1939, **13**, 195.

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EFFECTS OF GUAIACOL AND HEXYLRESORCINOL IN THE PRESENCE OF BARIUM AND CALCIUM

BY W. J. V. OSTERHOUT

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(Received for publication, April 18, 1940)

Höber[1] holds that alkaline earths affect the colloids of protoplasm so as to inhibit the depolarizing effect of K^+. Guttman[2] states that they prevent the depolarizing action of various organic substances.

These conclusions, based on experiments with muscle and nerve, have been tested on *Nitella*. In a previous paper it is shown that in some cells the effect of K^+ is partly inhibited[3] by Ca^{++} when the concentration of K^+ is 0.01 M but not when it is 0.1 M. In other cells no such inhibition occurs. The present paper shows that Ca^{++} and Ba^{++} do not inhibit the depolarizing effects of guaiacol and hexylresorcinol.

The depolarizing action of guaiacol has been described in previous papers.[4] The normal positive[5] P.D. of about 100 mv. decreases (*i.e.*, changes in a negative direction) when guaiacol is applied and if the concentration is not too high the process is reversible.

In order to test the effect of Ca^{++} on this process the following experiments were made.[6]

In all the experiments two spots on the same cell, *D* and *E*, were connected through the recording galvanometer to a spot *F* (which was in

[1] Höber, R., and Strohe, H., *Arch. ges. Physiol.*, 1929, **222**, 71. Höber, R., Andersh, M., Höber, J., and Nebel, B., *J. Cell. and Comp. Physiol.*, 1939, **13**, 195.

[2] Guttman, R., *J. Gen. Physiol.*, 1939–40, **23**, 343.

[3] Osterhout, W. J. V., and Hill, S. E., *J. Gen. Physiol.*, 1938–39, **22**, 139.

[4] Osterhout, W. J. V., *J. Gen. Physiol.*, 1938–39, **22**, 417; 1939–40, **23**, 171.

[5] The P.D. is called positive when the positive current tends to flow from the sap across the protoplasm to the external solution.

[6] The cells, after being freed from neighboring cells, stood in the laboratory at 15° ± 1°C. in Solution A (*cf.* Osterhout, W. J. V., and Hill, S. E., *J. Gen. Physiol.*, 1933–34, **17**, 87) for several days. They belonged to Lot B (*cf.* Hill, S. E., and Osterhout, W. J. V., *Proc. Nat. Acad. Sc.*, 1938, **24**, 312).

The measurements were made on *Nitella flexilis*, Ag., using the technique described in former papers (Hill, S. E., and Osterhout, W. J. V., *J. Gen. Physiol.*, 1937–38, **21**, 541). Unless otherwise stated the changes here recorded were reversible and there were no signs of injury.

contact with 0.01 M KCl and in consequence had a P.D. which remained constant, approximately at zero, during the experiment).

In one set of cells the spot *D* was at first in contact with 0.01 M NaCl: when this was replaced by 0.01 M NaCl + 0.02 M guaiacol there was a loss[7] of P.D. amounting to 103 ± 11.9 mv. (6 observations on 6 cells). When 0.01 M NaCl in contact with *E* was replaced by 0.01 M $CaCl_2$ there was a loss[7] of P.D. amounting to 16.5 ± 1 mv. (6 observations on 6 cells). Then 0.01 M $CaCl_2$ was replaced by 0.01 M $CaCl_2$ + 0.02 M guaiacol which entailed a further change of P.D. in a negative direction[8] amounting[4] to 115 ± 12.5 mv. (5 observations on 5 cells).

The experiment was repeated on another set of cells, using Ba^{++} in place of Ca^{++}. The result was similar. Addition of 0.02 M guaiacol to 0.01 M NaCl at *D* caused a change of P.D. of 108 ± 11.9 mv. in a negative direction (5 observations on 5 cells). Replacing 0.01 M NaCl by 0.01 M $BaCl_2$ at *E* caused a rapid loss of P.D. amounting to 25 ± 2.3 mv. (5 observations on 5 cells). Replacing 0.01 M $BaCl_2$ by 0.01 M $BaCl_2$ + 0.02 M guaiacol caused a change of P.D. in a negative direction amounting to 107 ± 6.6 mv. (5 observations on 5 cells).

Experiments with hexylresorcinol[9] gave similar results. For example, a set of experiments was made in which 0.01 M NaCl at *D* was replaced by 0.01 M NaCl + 0.0003 M hexylresorcinol, giving a change[10] of P.D. in a negative direction of 104 ± 10.4 mv. (6 observations on 6 cells). When 0.01 M $CaCl_2$ at *E* was replaced by 0.01 M $CaCl_2$ + 0.0003 M hexylresorcinol the change of P.D. was 106 ± 10.5 mv. (6 observations on 6 cells).

In another set of cells 0.001 M NaCl was placed at *D*. When this was replaced[11] by 0.015 M NaCl there was a loss of P.D. of 30 ± 1.2 mv. (8 observations on 8 cells). This solution was then replaced by 0.015 M NaCl + 0.0003 M hexylresorcinol, causing a further loss of 79 ± 7.4 mv. (8 observations on 8 cells).

Then 0.001 M NaCl at *E* was replaced by 0.001 M NaCl + 0.01 M $BaCl_2$ (thus making the osmotic pressure about the same as that of 0.015 M NaCl

[7] This took place in a few seconds, indicating that the effect was chiefly at the outer protoplasmic surface.

[8] This was a gradual change in a negative direction (depolarization) involving an action current in some cases.

[9] The hexylresorcinol was kindly donated by the firm of Sharp and Dohme, Glenolden, Pennsylvania.

[10] Osterhout, W. J. V., *J. Gen. Physiol.*, 1939-40, **23**, 569.

[11] The object of this was to make the osmotic pressure about the same as in the subsequent experiment at *E*.

at D): this caused a loss of P.D. of 45 ± 2.8 mv. (8 observations on 8 cells). When this was replaced by 0.001 M NaCl + 0.01 M $BaCl_2$ + 0.0003 M hexylresorcinol there was a further change of P.D. in a negative direction amounting to 106 ± 3.5 mv. (8 observations on 8 cells).

It is evident that the depolarizing effect of guaiacol and of hexylresorcinol is not inhibited by Ca^{++} or Ba^{++}. It is possible that this may be due to the fact that Ca^{++} and Ba^{++} do not penetrate to the inner protoplasmic surface which is the chief seat[12] of the P.D. and which is reached by guaiacol and hexylresorcinol.

The effects of these organic depressants come on slowly, indicating that they penetrate gradually to the inner protoplasmic surface. But when NaCl is followed by $CaCl_2$ or by $BaCl_2$ the change in P.D. is immediate, indicating that their effects are chiefly at the outer protoplasmic surface.

SUMMARY

Guaiacol was applied at two spots on the same cell of *Nitella*. At one spot it was dissolved in 0.01 M NaCl, at the other in 0.01 M $CaCl_2$ or $BaCl_2$. The effect was practically the same in all cases, *i.e.* a similar change of P.D. in a negative direction, involving a more or less complete loss of P.D. (depolarization).

When hexylresorcinol was used in place of guaiacol the result was similar.

That Ca^{++} and Ba^{++} do not inhibit the effect of these organic depolarizing substances may be due to a lack of penetration of Ca^{++} and Ba^{++}. The organic substances penetrate more rapidly and their effect is chiefly on the inner protoplasmic surface which is the principal seat of the P.D.

[12] Osterhout, W. J. V., *J. Gen. Physiol.*, 1934–35, **18**, 215.

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ACTION CURVES WITH SINGLE PEAKS IN NITELLA IN RELATION TO THE MOVEMENT OF POTASSIUM

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(Received for publication, April 17, 1940)

Although closely related, *Chara*[1] and *Nitella*[2] show striking differences. The action curve in *Nitella* has two peaks but in *Chara* there is only one. The outer protoplasmic surface in *Nitella* is sensitive to K^+ but this is not true of *Chara*.

It is of decided interest to find that these differences can be abolished by appropriate treatment. When cells of *Nitella* are leached in distilled water the outer surface becomes insensitive[3] to K^+ and at the same time the form of the action curve approaches that of *Chara* and shows only a single peak. The treatment with distilled water[3] commonly removes the irritability[4] as well as the sensitivity[5] to K^+ but cells are occasionally met with in which the irritability persists after the sensitivity to K^+ disappears. These present some interesting features.

Under normal conditions, cells of *Nitella* have an outwardly directed (positive[6]) P.D. of about 100 mv. due chiefly to the outwardly directed concentration gradient[7] of K^+ across the inner protoplasmic surface Y.

When an action current appears this P.D. disappears, partially or completely, producing the first movement of the action curve, *i.e.* the spike, or *o* movement, as seen in Fig. 1.

This is presumably due to an increase in the permeability of Y which

[1] *Chara coronata*, Ziz. The large cells, resembling those of *Nitella*, are not covered with a layer of small cells as in most species of *Chara*.

[2] *Nitella flexilis*, Ag.

[3] Osterhout, W. J. V., and Hill, S. E., *J. Gen. Physiol.*, 1933–34, **17**, 105.

[4] By this is meant the ability to give propagated action currents on electrical stimulation.

[5] By this is meant the large change in P.D. when 0.01 M KCl is replaced by 0.001 M KCl or by 0.01 M NaCl (the latter is called for convenience the potassium effect).

[6] The P.D. is called positive when the positive current tends to flow from the sap across the protoplasm to the external solution.

[7] Since the effect of K^+ predominates the other cations are omitted from the discussion. *Cf.* Osterhout, W. J. V., *J. Gen. Physiol.*, 1934–35, **18**, 215.

allows K+ to migrate outward in the form of a moving boundary.[8] This destroys the concentration gradient of K+ across Y and produces a loss of P.D.

On reaching X (when X is sensitive to K+) K+ will set up an outwardly directed (positive) potential, causing the curve to fall and producing the p movement of the first peak. This movement will last until K+ reaches the outer surface of X and thus diminishes the concentration gradient of K+ across X.

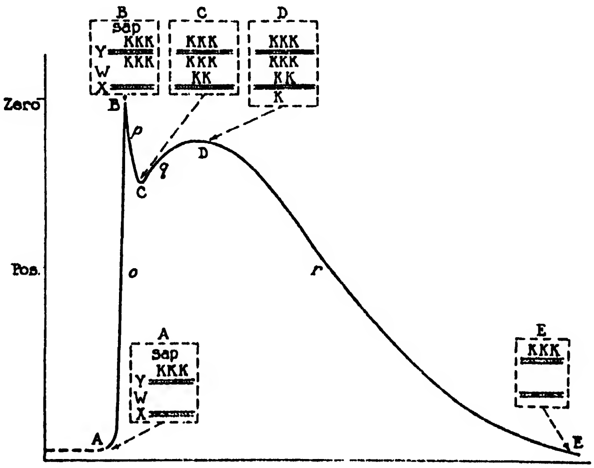

FIG. 1. The unbroken line shows changes in P.D. during the action current in *Nitella*, supposedly due to the outward movement of potassium. The broken line shows the P.D. in the resting state, before the outward movement of potassium begins.

In the diagrams the symbol K denotes the outwardly moving potassium (reduction in concentration is shown by reduction in the number of symbols). Each stage of its progress is marked by a change in P.D.: for example, in Diagram A the observed P.D. is due to the relatively high concentration of potassium at the inner surface of Y; in Diagram B we see that potassium has reached the outer surface of Y and in consequence the P.D. has disappeared.

The duration of the action current is usually about 15 seconds.

The duration of this downward movement will depend on the speed with which K+ moves across X. Its magnitude will depend on the sensitivity of X to K+, *i.e.* on the mobility ratio[9] $u_K \div v_{Cl}$, and on the partition coefficient S_K (S_K = concentration of K+ in the non-aqueous protoplasmic surface ÷ concentration of K+ in the adjacent aqueous solution).

It will be larger when the moving boundary is sharp for then the concen-

[8] Regarding moving boundary see MacInnes, D. A., and Longsworth, L. G., *Chem. Rev.*, 1932, **11**, 171.

[9] *Cf.* Osterhout, W. J. V., and Hill, S. E., *J. Gen. Physiol.*, 1938–39, **22**, 139.

tration gradient of K^+ across X will be larger. If the front of the moving boundary becomes more diffuse the concentration of K^+ as it strikes X will diminish and if it scarcely exceeds the concentration of K^+ already present in W the p movement will practically disappear.

This may explain such a curve as is seen[10] in Fig. 2 which may occur even when X is sensitive to K^+. Such curves are occasionally found, especially in cells exposed to 0.01 M NaCl[11] which may increase the protoplasmic motion and render the moving boundary more diffuse. In such cases it is no longer possible to distinguish sharply between the fall of the curve due to the p movement and the fall due to the r movement (*i.e.* to the recovery which is presumably due to the movement of K^+ back into the sap). In such cases the only evidence of the p movement lies in a sudden change in the course of the curve, as in Fig. 2.

If the outer protoplasmic surface X loses part of its sensitivity to K^+ the p movement will fall off in consequence.

When X has lost its sensitivity to K^+ (as shown by the absence of the potassium effect[12]) there is no longer any reason to expect any abrupt change in the course of the curve.[13]

FIG. 2. Action curve in an unleached cell of *Nitella* with normal potassium effect; *i.e.*, with the outer protoplasmic surface sensitive to K^+. The first peak is missing, presumably because the outwardly moving K^+ has a diffuse rather than a sharp boundary.

The spot recorded, D, is in contact with 0.0001 M NaCl; it is connected through the recording galvanometer to a spot F which is in contact with 0.01 M KCl and consequently has a constant P.D. approximately at zero. Hence the action curve is monophasic.

The cell was freed from neighboring cells and kept for 10 days in Solution A at 15 ± 1°C. The record was made at 22°C. (electrical stimulation). Vertical marks 5 seconds apart.

[10] The cells, after being freed from neighboring cells, stood in the laboratory at 15° ± 1°C. in Solution A (*cf.* Osterhout, W. J. V., and Hill, S. E., *J. Gen. Physiol.*, 1933–34, **17,** 87) for several days.

The measurements were made on *Nitella flexilis*, Ag., using the technique described in former papers (Hill, S. E., and Osterhout, W. J. V., *J. Gen. Physiol.*, 1937–38, **21,** 541).

[11] *Cf.* Hill, S. E., and Osterhout, W. J. V., *J. Gen. Physiol.*, 1938–39, **22,** 91. It is here suggested that increased conductivity of the protoplasm may tend to produce single peaks.

[12] *I.e.* when there is no change in P.D. on replacing 0.01 M KCl by 0.01 M NaCl.

[13] At the start of the spike there may be an abrupt rise of the curve due to electrical leakage from the stimulating electrodes.

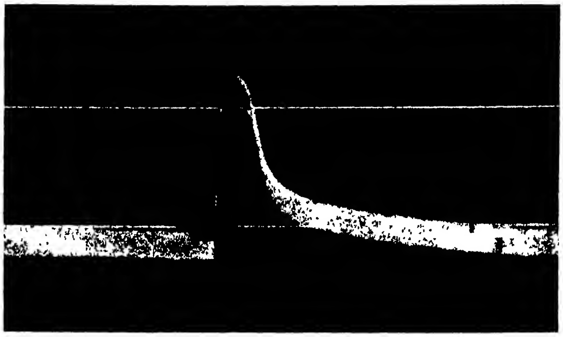

FIG. 3. Action curve in a cell of *Nitella* which has lost its potassium effect (*i.e.* the outer protoplasmic surface has become insensitive to K^+) as the result of leaching in distilled water.

The spot recorded, *D*, was in contact with 0.01 M NaCl and was connected through the recording galvanometer with another spot, *F*, in contact with 0.01 M NaCl (the P.D. of the latter remained constant as evidenced by the record of another spot *E* not shown here). In consequence the action curve is monophasic.

The cell was freed from neighboring cells and kept for 8 days in Solution A, then for 5 days in distilled water: the temperature was 15 ± 1°C. The record was made at 23°C. (electrical stimulation). Vertical marks 5 seconds apart.

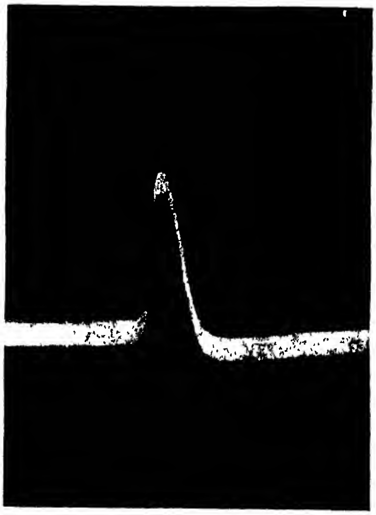

FIG. 4. Action curve in a normal cell of *Chara* which shows no potassium effect; *i.e.*, the outer protoplasmic surface is not sensitive to K^+.

The spot recorded, *D*, was in contact with 0.001 M KCl: it was connected through the recording galvanometer with a spot *F* killed by chloroform and having in consequence a P.D. of zero. Hence the action curve is monophasic.

The cell was freed from neighboring cells and kept for 2 days in Solution A at 15 ± 1°C. The record was made at 24°C. Vertical marks 5 seconds apart.

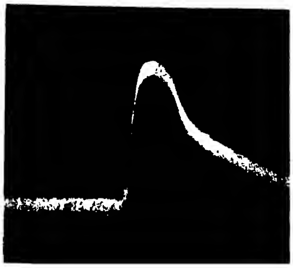

FIG. 5. Action curve in a cell of *Nitella* which has lost the potassium effect (*i.e.* the outer protoplasmic surface has become insensitive to K^+) as the result of leaching in distilled water.

The spot recorded, *D*, was in contact with 0.01 M NaCl: it was connected through the recording galvanometer with another spot *F* in contact with 0.01 M NaCl which had a constant P.D. during the record as evidenced by the record of another spot *E* (not shown here). In consequence the action curve is monophasic.

The cell was freed from neighboring cells and kept for 12 days in Solution A and then placed in distilled water for 7 days: the temperature was 15 ± 1°C. The record was made at 22°C. Vertical marks 5 seconds apart.

FIG. 6. Action curve in a cell of *Nitella* which has lost the potassium effect (*i.e.* the outer protoplasmic surface has become insensitive to K^+) as the result of leaching in distilled water.

The spot recorded, *D*, was in contact with 0.01 M NaCl: it was connected through the recording galvanometer to another spot *F* in contact with 0.01 M NaCl, which had a constant P.D. during the record, as evidenced by the record of another spot *E* (not shown here). Hence the action curve is monophasic.

The cell was freed from neighboring cells and kept in distilled water for 6 days at 15 ± 1°C. The record was made at 23°C. Heavy vertical marks 5 seconds apart.

Under these circumstances we expect only curves with rounded tops,[14, 15]

[14] Since there is no *p* movement there is no *q* movement.

[15] This applies to action curves in which the P.D. is largely lost and the spike or *o* movement follows the usual course and goes nearly to zero. When only a relatively small loss of P.D. occurs rounded tops may occur even when *X* is sensitive to K. Such action curves are regarded as abnormal and probably involve only a small outward movement of K^+ which may not reach *X* at all or only to a slight extent. See Osterhout, W. J. V., *Biol. Rev.*, 1931, **6,** 369 (Fig. 12 b).

such as are actually observed[16] under these conditions (Fig. 3). Fig. 3 may be compared with the normal curve[17] of *Chara* (Fig. 4).

The course of recovery may be shorter than is usually observed in *Nitella* but this is not always the case, as is evident from Figs. 5 and 6.

These facts strongly support the suggestion previously made[18] that K^+ plays an important rôle in the action curve.

SUMMARY

In *Nitella* the action curve has two peaks, apparently because both protoplasmic surfaces (inner and outer) are sensitive to K^+.

Leaching in distilled water makes the outer surface insensitive to K^+. We may therefore expect the action curve to have only one peak. This expectation is realized.

The action curve thus obtained resembles that of *Chara* which has an outer protoplasmic surface that is normally insensitive to K^+.

The facts indicate that the movement of K^+ plays an important part in determining the shape of the action curve.

[16] In some cases the potassium effect may be restored by pressure on the cell (Hill, S. E., and Osterhout, W. J. V., *J. Gen. Physiol.*, 1934–35, **18,** 687) or by an action current (Osterhout, W. J. V., and Hill, S. E., *J. Gen. Physiol.*, 1934–35, **18,** 681). Hence a cell showing an action curve with one peak may sometimes show a potassium effect when tested later on.

When a cell is tested for the potassium effect with negative result but when subsequently stimulated shows a tendency to form a second peak this may be due to the fact that the action current tends to restore the potassium effect and consequently the double peak.

[17] After the spike the curve in Fig. 4 falls below the original level (positive after potential) and then rises. This is not a constant feature of *Chara* and it may also occur in *Nitella*.

In *Chara* the chief seat of the P.D. appears to be (as in *Nitella*) the inner protoplasmic surface Y which is presumably sensitive to K^+ although the outer surface, X, is not. Leached *Nitella* resembles *Chara* in that Y is sensitive to K^+ but X is not.

[18] Osterhout, W. J. V., *J. Gen. Physiol.*, 1934–35, **18,** 215.

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SEPARATION OF POTASSIUM ISOTOPES IN VALONIA AND NITELLA

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(Received for publication, April 15, 1940)

A. K. Brewer[1] reports that in certain cases the ratio $K^{39} \div K^{41}$ in the organism is not the same as in the environment. This situation appears to exist in *Valonia*[2] and *Nitella*[3] according to determinations made by Dr. Brewer to whom the author has sent samples of sap. The author desires to make grateful acknowledgment of Dr. Brewer's kindness.

The cells of *Valonia* were removed from the sea water, rinsed in distilled water, and pierced by a glass tube tapered to a fine capillary. By means of gentle pressure or suction the tube was filled with sap which was transferred to quartz or Pyrex bottles.

The *Nitella* cells were rinsed with distilled water and cut open to allow the sap to flow out into quartz or Pyrex vessels.

The results are shown in Table I. In the opinion of Dr. Brewer, they indicate some separation of K^{39} from K^{41} by *Valonia* and *Nitella*.

It is evident that this separation cannot be due to diffusion. The diffusion path in both cases is very short.

It seems possible that the separation depends on a higher partition coefficient, S, for K^{41} than for K^{39} (S = concentration in the non-aqueous protoplasmic surface layer $\div$ concentration in the external solution). A similar explanation has been suggested[4] for the fact that K^+ is taken up by *Valonia* in preference to Na^+ and this can be imitated to a certain extent by guaiacol which takes up more K^+ than Na^+. This is in accordance with the rule of Shedlovsky and Uhlig[5] which states that the partition coefficient increases with the ionic radius. On this basis we might expect K^{41} to be taken up more than K^{39}.

* Work completed by Dr. Jacques before his death in February, 1939.

[1] Brewer, A. K., *J. Am. Chem. Soc.*, 1936, **58**, 365, 370; *Ind. and Eng. Chem.*, 1938, **30**, 893. Lasnitzki, A., and Brewer, A. K., *Nature*, 1938, **142**, 538.

[2] *Valonia macrophysa*, Kütz., collected in Bermuda.

[3] *Nitella flexilis*, Ag., collected in the vicinity of New York City.

[4] *Cf.* Osterhout, W. J. V., *Bot. Rev.*, 1936, **2**, 283.

[5] Shedlovsky, T., and Uhlig, H. H., *J. Gen. Physiol.*, 1933–34, **17**, 549, 563.

In view of this the following experiment was made. A solution of 0.2 M KOH was shaken with a mixture of 70 per cent guaiacol + 30 per cent *p*-cresol: this will be called G.C. mixture for convenience. The K^+ was extracted from the G.C. mixture by shaking with 0.5 M H_2SO_4 and was reconverted to KOH: this was shaken with fresh G.C. mixture; from this the K^+ was again removed by H_2SO_4, converted to KOH, and again shaken with G.C. mixture, and so on. After 13 repetitions[6] the ratio of $K^{39} \div K^{41}$ in the G.C. mixture was 14.10 ± 0.04. At the start it was 14.20 ± 0.04. Dr. Brewer regards this change as possibly significant.

TABLE I

Solution		$K^{39} \div K^{41}$
Bermuda sea water		14.20 ± 0.03
Valonia sap	Sample I	13.85 ± 0.05
	Sample II	13.85 ± 0.05
Nitella sap	Sample I	13.85 ± 0.03
	Sample II	14.00 ± 0.03
KOH before shaking		14.20 ± 0.02
K taken up by guaiacol mixture	Sample I	14.10 ± 0.04
	Sample II	14.09 ± 0.03

This result suggests that further experiments with organic substances may prove interesting.

SUMMARY

The ratio of $K^{39} \div K^{41}$ appears to be lower in the sap of *Valonia* and *Nitella* than in the environment, indicating that the living cell can separate these isotopes to some extent.

Experiments with a mixture of guaiacol and *p*-cresol suggest that a similar separation may occur here but further experiments are needed.

[6] A similar result (decrease of the ratio $K^{39} \div K^{41}$ in the G.C. mixture) would presumably be achieved by shaking the G.C. mixture once with a sufficiently large volume of aqueous KOH.

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THE PURIFICATION OF CATHEPSIN

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(*From the Laboratories of The Rockefeller Institute for Medical Research, Princeton, New Jersey*)

(Received for publication, February 23, 1940)

The Cathepsin System.—Extracts of animal tissues contain a proteolytic system which is active at pH 3.5. This system consists of a proteinase part called "cathepsin" which initiates the digestion of protein, and a peptidase part which carries the digestion further. The activity of the tissue peptidase can be increased by cysteine and abolished by iodoacetic acid. The activity of the tissue proteinase, however, is not affected by these reagents (Anson, 1937). To estimate the proteinase alone denatured hemoglobin is digested for a short time and to a slight extent at pH 3.5, the undigested protein is precipitated by *dilute* (0.19 N) trichloracetic acid, and the unprecipitated digestion products are estimated by the color they give with the phenol reagent (Anson, 1937, 1938).

Fruton and Bergmann (1939) have now found synthetic peptide substrates for two different tissue enzymes. One of these enzymes is activated by cysteine, the other is not.

The Function of Cathepsin.—Cathepsin is the only proteinase known to be present in animal cells and unicellular organisms generally. Those cells which are particularly active in the synthesis of proteins are particularly rich in cathepsin. The optimum pH for the digestion of proteins by tissue cathepsin is 3.5, whereas living tissues are much more alkaline. There is, at present, no direct evidence concerning the function of cathepsin in living cells. The most likely hypothesis is that cathepsin catalyzes the synthesis of cellular proteins.

Crude extracts of animal tissue which contain a mixture of proteolytic enzymes carry the digestion of protein very far. It would hardly be expected that such extensive breakdown could be reversed *in vitro*. Thus, the first step in experiments on *in vitro* synthesis of protein is the preparation of cathepsin as free from other proteolytic enzymes as possible.

The Purification of Cathepsin.—This paper describes the purification of cathepsin extracted from beef spleen. 1 mg. of the protein in the purified preparation has the same activity as the extract of 1.3 gm. of spleen. Some

inert protein is first destroyed by autolysis. More inert protein is removed by adsorption first by spleen material and then by aluminum hydroxide. Finally, the cathepsin and most of the remaining inert protein is precipitated from a very dilute solution by tungstic acid. The tungstic acid precipitate of cathepsin is stable in the cold.

The specific activity of the protein precipitated by tungstic acid can be increased at least 8 times by extraction of the tungstic acid precipitate with barium hydroxide plus barium chloride and by the application to this extract of further procedures which have not yet been standardized and so will not be described in this paper.

Purified cathepsin is not inactivated when exposed to 0.01 M iodoacetamide for an hour at pH 8.0 and at 25°C. When added to gelatin at pH 3.5 in the presence of cysteine it does not cause a detectable increase in the formol titration. The experiments with gelatin were not carried out with the cathepsin preparation described in this paper.

The Starting Material.—Beef spleen is used as a source of cathepsin because it is relatively rich in cathepsin and relatively cheap. Even spleen, however, contains extremely little proteinase compared with specialized tissues such as exist in the stomach and pancreas. Thus, the extract of 1 gm. of beef spleen has 3.7×10^{-3} hemoglobin units of cathepsin activity whereas the extract of 1 gm. of pig stomach fundus mucosa has 5×10^{-1} hemoglobin units of pepsin activity.

The Purification Procedure.—The following outline of the procedure used for the purification of cathepsin states in a general way what the various steps are, what they accomplish, and in what way they are of general interest for the methodology of the purification of tissue proteins. Detailed directions are given in the experimental part.

1. Frozen beef spleen is thawed and suspended in water for a day. During this procedure some autolysis takes place. About half the nitrogen of the spleen is extracted and about half the nitrogen in the extract is protein nitrogen.

2. The suspension is 0.3 saturated with ammonium sulfate, acidified to green to brom cresol green, and heated to 45°C. Extensive further autolysis takes place, denatured protein is precipitated, and the mass of insoluble material is clotted, which facilitates filtration. The bulky mass of insoluble material adsorbs the cathepsin as well as practically all the unautolyzed other protein present. When the acidified and heated spleen material is filtered most of the products of autolysis remain in the filtrate and are thus removed.

Cathepsin not adsorbed to insoluble spleen material is unstable at 45°C.

in acid solution. Thus the adsorption protects the cathepsin from destruction under the conditions which bring about extensive autolysis. Purified cathepsin not adsorbed to insoluble spleen material is soluble in 0.3 saturated ammonium sulfate. Thus the adsorption also makes possible the separation of cathepsin from split products formed during autolysis.

3. The insoluble mass remaining after filtration of the acid autolysate is made slightly alkaline (red to phenol red) with a sodium hydroxide solution of the amphoteric aluminum hydroxide. The aluminum hydroxide which is soluble only in strong alkali is precipitated again at red to phenol red and helps with the filtration. It is not present in sufficiently large amount to be important as an adsorbent. The insoluble spleen material which adsorbed all the cathepsin in the acid ammonium sulfate solution no longer adsorbs cathepsin at red to phenol red and so after filtration the cathepsin is all obtained in the filtrate. Some inert protein, however, remains adsorbed to the insoluble spleen material and thus a purification as well as an extraction of the cathepsin is obtained by the addition of alkali. If the suspension is made too alkaline less inert protein is removed. If the solution is made too acid some cathepsin is carried down with the precipitate.

4. Some inert protein in the alkaline filtrate and about 25 per cent of the cathepsin are removed by adsorption with aluminum hydroxide at red to phenol red. If more than the recommended aluminum hydroxide is used or the pH is made less alkaline, more cathepsin is lost. If the amount of aluminum hydroxide is decreased or the pH made more alkaline, less inert protein is removed and a product of lower specific activity obtained. This procedure, like most adsorptions by hydroxide gels, is sensitive to the exact conditions and so does not give completely reproducible results.

Instead of adding previously prepared aluminum hydroxidc, as is usually done, aluminum hydroxide in the procedure just described is formed in the spleen extract by the addition first of partially neutralized aluminum chloride and then of sodium hydroxide. Aluminum hydroxide formed in the extract removes much more inert protein than the same amount of aluminum hydroxide prepared before being added to the extract. Presumably protein does not penetrate rapidly into the interior of an aluminum hydroxide gel.

5. The cathepsin and most of the remaining protein and a part of the protein split products are precipitated by tungstic acid at green to brom cresol green. At this pH the proteins are precipitated by tungstic acid in the native form. When tungstic acid is used to remove proteins for analyti-

cal work on protein-free filtrates, the protein is usually denatured as the result of the solution being made too acid. So tungstic acid has in general not been used for preparative work. Tungstic acid is chosen for the precipitation of cathepsin despite the fact that it precipitates some of the protein split products. It is the only reagent I have found which will precipitate cathepsin even when the cathepsin is present in an extremely dilute solution containing much more protein split products than protein and which is cheap enough to be used for very large volumes of solutions. The presence of tungstate, furthermore, is useful in the further purification of cathepsin with barium hydroxide.

TABLE I

Purification of Cathepsin

	Hb units per gm. spleen	Hb units per mg. protein N	Hb units per mg. tyrosine protein color value	Hb units per mg. tyrosine total color value
	$\times 10^4$	$\times 10^4$	$\times 10^4$	$\times 10^4$
Filtrate from autolyzed spleen suspension	37	4.6	11.8	6.5
Alkaline extract of acidified and heated spleen	18		94	17.5
Alkaline extract purified with $Al(OH)_3$	13.5	114	330	22
Tungstic acid precipitate of purified extract	12			120

Many techniques which are used in the fractionation of proteins are not practical for large scale work with tissues. Yet if many experiments are to be done on the purification of an enzyme which is present in low concentrations large scale work is necessary. The procedure for the purification of cathepsin which has been outlined permits one man to work up as much as 40 kilograms of spleen at a time with very simple apparatus and can also be applied to small quantities of spleen.

Results.—Table I gives a typical series of results. The first column shows the amount of proteinase left at each step per gram of spleen starting material. About half the loss of proteinase is a purely mechanical loss due to the fact that in the alkaline extraction the precipitate is not washed. The other columns give the activities per milligram protein nitrogen and per unit total and protein color value. By protein is meant the material precipitated by 0.2 N trichloracetic acid. The protein content of the tungstic acid precipitate is not given because protein cannot be estimated in the presence of tungstic acid which precipitates protein split products.

The color value is the color given with the phenol reagent expressed as the number of milligrams of tyrosine which give the same color. The color value is easier to measure than the nitrogen value and it can be measured in the presence of ammonium sulfate. Purification of the cathepsin changes the protein color value per milligram of protein nitrogen only slightly. Although activity, nitrogen, and color values are given for the filtrate from the suspension of thawed spleen, in practice this suspension is not filtered but is acidified and heated.

Note on Maver's Experiments.—Maver (1939) digested denatured hemoglobin at pH 3.5 for 3 hours at 37°C. with a somewhat purified liver extract and measured by the Kjeldahl method the amount of protein precipitated by trichloracetic acid both before and after digestion. She found that the amount of protein changed into a non-precipitable form was increased 3.8 times by the addition of cysteine and concluded that the proteinase, cathepsin, is activated by cysteine. The experimental conditions used by Maver differ in two important respects from the conditions used in my hemoglobin method. The trichloracetic acid is 1 N instead of 0.187 N and the digestion is carried on much longer and much further than in my original experiments.

The conditions used by Maver, apart from being inconvenient, are not suitable for the estimation of proteinase in the presence of peptidase. I have found that for a given amount of digestion the nitrogen content of the trichloracetic acid filtrate of a hemoglobin digest is 1.5–3.0 times less when the trichloracetic acid concentration is 1 N than when it is 0.2 N. The exact difference depends on the extent and conditions of digestion.[1] Hiller and Van Slyke (1922) showed that 10 per cent trichloracetic acid precipitates somewhat more of Witte's peptone than 2.5 per cent trichloracetic acid. Thus, under Maver's conditions one estimates not only the initial digestion of protein due to proteinase but also the further digestion of split products precipitable by concentrated but not by dilute trichloracetic acid which may be due to peptidase. Secondly, in the estimation of proteinase it is not desirable to carry the digestion far since extensive digestion introduces complications due to the inhibition of proteinase by the products of digestion. When the extent of digestion of protein is great the extent of digestion is no longer sensitive to the amount of enzyme used and an apparent activation of proteinase may really be an activation of peptidases which digest split product inhibitors.

I have digested hemoglobin for 3 hours with a crude spleen extract, added trichloracetic acid, filtered off the precipitate, and estimated by the Kjeldahl method the nitrogen

[1] This observation suggests a method for preparing large split products from proteins. If the protein is digested *slightly* and the undigested protein is precipitated by 0.2 N trichloracetic acid and removed by filtration, then most of the material in the filtrate can be precipitated by the addition of more trichloracetic acid. The exact concentration of trichloracetic acid for maximum precipitation should be determined empirically, since the amount precipitated decreases again if the trichloracetic acid is made too concentrated. 1 N trichloracetic acid was used in the present experiments only because it was used by Maver. As digestion proceeds the fraction of the material in the filtrate precipitable by the addition of more trichloracetic acid becomes less.

content of the filtrate. When a small amount of enzyme was used and the final concentration of trichloracetic acid was 0.2 N then the nitrogen content of the filtrate (corrected for the blank) was 1.22 times as great when cysteine was present during the digestion than when cysteine was not added. When, however, 12 times as much enzyme was used and the final trichloracetic acid concentration was 1 N then the filtrate contained 1.54 times more nitrogen if cysteine was present than if it was not (Table II).

I do not know the reason for the small and unimportant cysteine effect obtained even when the extent of digestion is small and dilute trichloracetic acid is used. There may be some inactivation of cathepsin in the 3 hour digestion period used by Maver, which inactivation is inhibited by cysteine. I have already shown that such inactivation and inhibition can take place in more acid solution (Anson, 1937). There may be some small salt effect such as can also be obtained with ammonium sulfate. In any case, there is no evidence that the small cysteine effect is due to activation of enzyme at all. Enzymes of the papain type are usually almost completely inactivated by much handling and the cysteine effect is usually very great.

TABLE II

Effect of Cysteine on Digestion of 100 Mg. Hemoglobin

Amount crude enzyme used for digestion mg. N	Presence of cysteine	Final normality of trichloracetic acid	Mg. N in total 17 cc. trichloracetic acid filtrate—corrected for blank
0.068	−	0.2	0.86
0.068	+	0.2	1.04
12 × 0.068	−	1.0	2.7
12 × 0.068	+	1.0	4.0

The somewhat larger cysteine effect obtained when, following Maver, extensive digestion and concentrated trichloracetic acid are used is probably due to an activation of peptidase. Tissue peptidase is activated by cysteine (Anson, 1937) and, as has already been pointed out, under Maver's conditions the hemoglobin-trichloracetic acid method may well measure peptidase in addition to proteinase.

The present experiments do not explain why Maver observed a 280 per cent increase in the nitrogen content of the trichloracetic acid filtrate when I observed only a 54 per cent increase. The two enzyme preparations were different and perhaps Maver's enzyme preparation did contain, as she concluded, a proteinase activated by cysteine.

In conclusion, when conditions are chosen under which proteinase alone is measured, no large cysteine effect is observed even when a long digestion period is used and the split products are estimated by the Kjeldahl method. The large cysteine effect reported by Maver cannot be accepted as an activation of proteinase until it is shown that under Maver's conditions only proteinase is estimated.

EXPERIMENTAL

Estimation of Cathepsin.—Cathepsin is estimated as previously described (Anson, 1938). The preparation of the hemoglobin substrate has, however, been modified by the treatment of the red blood corpuscles with toluol (Anson, 1939). It is necessary that the empirical directions be followed rigorously if it is desired to express the activities

measured in terms of the standard hemoglobin units. The following precautions should be taken in preparing the reagents and carrying out the procedure.

The first steps in preparing the hemoglobin substrate consist in centrifuging whipped blood, washing the red corpuscles with cold salt solution, and shaking the washed corpuscles with toluol. These steps should be carried out the same day the blood is obtained or, if this is not possible, the blood should be promptly cooled to 0°C., kept at 0°C., and worked up as soon as possible. If much bacterial multiplication takes place before the addition of toluol then split products are formed which are not completely removed by dialysis and, in addition, the substrate solution becomes contaminated with bacterial enzymes.

After the corpuscles have been shaken with toluol and the suspension is filtered, if there is a layer of toluol over the filtrate the filtrate is allowed to stand in a cylinder and the toluol is removed with a fine tipped syphon. Large amounts of toluol are not completely removed by the subsequent dialysis and toluol decreases the rate of digestion by cathepsin.

The dialyzed hemoglobin solution is stored frozen in cardboard or aluminum containers. These containers should be moisture tight so that the concentration of hemoglobin does not change with time. Wax paper should be placed between the covers of the containers and the containers and the joints on the bottom of the usual cardboard containers should be covered with paraffin.

When the hemoglobin solution is thawed for use only such an amount should be thawed as will be used in a week or two. This thawed hemoglobin solution is stored at 0-5°C. Eventually bacterial growth sets in. I have found no effective preservative which does not interfere with either the digestion or the colorimetric estimation of the products of digestion.

The dialyzed hemoglobin solution still contains a small amount of substances not precipitable with trichloracetic acid which give a color with the phenol reagent. This blank is measured from time to time by the method already described. If, as a result of bacterial action, the blank is high or rising the hemoglobin should be rejected rather than any attempt made to correct for the blank. The blank of the hemoglobin substrate prepared by the present procedure changes only slightly with storage of the hemoglobin until bacterial action finally sets in in the thawed solution.

After the hemoglobin has been partially digested by cathepsin the undigested hemoglobin is precipitated by the addition of 0.3 N trichloracetic acid, the suspension is filtered, and the filtrate is made alkaline with 0.5 N sodium hydroxide. It is important that the concentrations of trichloracetic acid and sodium hydroxide be checked by titration. Commercial solid trichloracetic acid contains varying amounts of water. A filter paper such as Whatman's No. 42 should be used which gives a clear filtrate and does not adsorb split products. The color value of the filtrate, as measured with the phenol reagent, should be the same as that of the supernatant solution obtained if the trichloracetic acid suspension is centrifuged.

To check the whole procedure the hemoglobin is digested with a standard cathepsin solution and the products of digestion estimated. A stock stable solution of cathepsin in 75 per cent glycerine is stored at 5°C. 1 cc. of this glycerine solution is removed with an Ostwald contain pipette and diluted to 15 cc. with water to give the standard solution used for digestion. Different batches of hemoglobin are digested at the same rate by a given amount of cathepsin and the hemoglobin can be stored frozen for 6 months without any effect on the rate at which it is digested.

Estimation of Protein.—The protein is precipitated by 0.2 N trichloracetic acid out of a solution diluted to contain about 0.3 mg. protein per cc. When the solution contains much protein split products it is necessary to leave the trichloracetic acid solution at the temperature of hot tap water for a few minutes and then cool it to room temperature. If the heating is omitted the precipitation of protein is very slow. If the cooling to room temperature is omitted some of the protein remains dissolved.

To estimate the protein colorimetrically, a trichloracetic acid precipitate containing about 3 mg. of protein is dissolved with 8 cc. of 0.5 N sodium hydroxide and 7 cc. of water. The color is then developed with 3 cc. of diluted phenol reagent as in the estimation of cathepsin (Anson, 1938). In the cathepsin procedure more sodium hydroxide is used because of the trichloracetic acid which has to be neutralized.

Purification Procedure.—Beef spleen is ground and frozen in one pound blocks at the slaughter house (Swift and Co., Chicago) and transported and stored frozen.

The frozen spleen is allowed to thaw in a cold room and then suspended in twice its weight of tap water and allowed to stand for at least 24 hours at room temperature with toluol (Eastman Practical) as a preservative. For small scale work the spleen is suspended in an aluminum milk pail (Sears Roebuck). For large scale work the spleen suspension is prepared in 60 liter aluminum kettles (Wear-Ever Semi-Heavy Stock Pot).

To each liter of suspension there are added 176 gm. of ammonium sulfate and, after the salt is dissolved, 70 cc. of 1 N HCl. During the addition of the acid the suspension is stirred vigorously by hand with a large wooden paddle such as can be obtained from hotel supply houses. Mechanical stirring results in hard packing of the insoluble spleen material.

The spleen suspension is heated to 45°C. with the aid of an aluminum coil through which 50–55°C. water is passed. A thermometer is introduced into the system with a Y-shaped aluminum connecting tube (Fischer Scientific Co., No. 15-321B) the thermometer being attached with thick rubber tubing. The suspension is stirred with the wooden paddle during the heating. It takes about 20 minutes to heat 35–40 liters of suspension with a 40 foot coil. The large kettle is covered with an aluminum cover and with blankets to prevent rapid cooling and is allowed to stand overnight. When a small amount of suspension is used it is transferred to a bottle which can be closed and is put away at 37°C.

The next morning the suspension is filtered on 50 cm. folded filter paper, the No. 612 sold in unfolded form by Eaton Dikerman Company, Mount Holly Springs, Pennsylvania. More suspension is poured on the papers when there is room, the final amount of suspension added to each funnel being about one and one-half times the amount needed to fill the funnel originally. After the filtration is about complete but before the solid material has begun to crack, the funnels are filled with tap water made green to brom cresol green with HCl, covered with aluminum covers (Sears Roebuck), and the filtration is allowed to continue overnight. More extensive washing is not possible without loss of cathepsin.

The next morning, the precipitates and the filter papers are stirred up with tap water, the total volume being that of the original spleen suspension before the addition of salt and acid. More toluol is added. The suspension is brought to red to phenol red by adding with stirring a solution made up of 1 part 2 M $AlCl_3$ and 10 parts 1 N NaOH. The suspension is stirred vigorously and more alkali is added as needed to keep the suspension red to phenol red.

After the suspension has been at red to phenol red for 1 hour it is poured on the large folded filter papers, covered, and allowed to filter overnight, no additional suspension being added to each paper. The precipitate is not washed although it occupies one third of the original volume. Washing is slow and results in a product with lower specific activity.

A partially neutralized $AlCl_3$ is made up by adding 1 N NaOH with mechanical stirring to an equal volume of 1 M $AlCl_3$. 40 cc. of this product are added to each liter of alkaline filtrate. 1 N NaOH is then added with mechanical stirring until the solution is red to phenol red and the $Al(OH)_3$ is filtered off with the aid of Hyflo Super-cel (Johns Manville) and suction on monel metal Buchner funnels of 33 cm. diameter (Louis Fuhro, New York City). The filter cake is washed with water made red to phenol red with NaOH until the total volume of the filtrate is about the same as the suspension before filtration.

Since the exact amount of $Al(OH)_3$ which gives considerable purification without too great loss of cathepsin is somewhat variable, it is safest, in large scale work, to find out by preliminary experiments with samples how much $Al(OH)_3$ gives about 25 per cent loss of cathepsin.

To each liter of filtrate, which must be at room temperature and not cold, there are added 10 cc. of 1 M technical sodium tungstate (Molybdenum Corporation of America) and with mechanical stirring enough 1 N HCl to make the solution green to brom cresol green. The suspension is allowed to stand 1 hour with occasional stirring. More HCl is added if necessary to keep the solution green to brom cresol green. The precipitate is filtered on large Buchner funnels with Hyflo Super-cel and is washed with 0.001 M tungstate made green to brom cresol green until the wash water gives the same sort of precipitate with a barium salt as the 0.001 M tungstic acid solution alone. When there is sulfate still present it is readily detected both by the amount and the character of the precipitate.

Finally the filter cake is suspended in water to give a suspension containing about 0.01 hemoglobin units of cathepsin per cc., assuming all the cathepsin to have been precipitated from the solution to which tungstic acid was added. Toluol is added as a preservative and the suspension is stored at 5°C. or frozen.

The Effect of Iodoacetamide on Cathepsin.—A tungstic acid precipitate of purified cathepsin is dissolved with sodium hydroxide. The final solution is red to phenol red and contains 2×10^{-4} hemoglobin units of cathepsin per cc. If the cathepsin is allowed to stand for 1 hour at 25°C. at red to phenol red in the presence of 0.01 M iodoacetamide no inactivation takes place.

The Effect of Cysteine on Digestion.—The hemoglobin substrate is made up without ammonium sulfate by adding 1 cc. of 1.35 M acetic acid to 4 cc. of 2.5 per cent dialyzed hemoglobin prepared as already described (Anson, 1938, 1939). To 5 cc. of this solution there is added either 0.5 cc. of 0.2 M cysteine HCl and 0.5 cc. of 0.16 N NaOH or in the control experiment 1 cc. of 0.1 N NaCl. 1 cc. of enzyme solution is then added and digestion is carried out for 3 hours at 37°C. 10 cc. of 0.34 N trichloracetic acid are added to give a final concentration of 0.2 N or 10 cc. of 1.7 N trichloracetic acid to give a final concentration of 1 N. The suspension is allowed to stand 2 hours before filtration. This may be a longer period than is needed to complete the precipitation but if the filtration of the concentrated trichloracetic acid suspension is carried out right away a precipitate gradually forms in the filtrate. The nitrogen content of 5 or 10 cc. filtrate is measured

by the Kjeldahl method. To measure the blank the substrate solution is left for 3 hours at 37°C. and the enzyme is added after the trichloracetic acid.

The enzyme is prepared by dialyzing a filtrate of the suspension of thawed spleen already described as the first step of the purification procedure. This crude preparation seems to be somewhat more active than the purified cathepsin used by Maver.

The results are shown in Table II.

SUMMARY

1. One mg. of the purified cathepsin whose preparation is described is as active as the extract of 1.3 gm. of spleen. An eightfold further purification is possible by procedures which are still being modified and so are not described in the present paper.

2. The first step of the purification consists in suspending frozen and thawed spleen in water and letting it autolyze.

3. In the second step, ammonium sulfate is added and the suspension is acidified and warmed. Much additional autolysis takes place. The cathepsin is protected from destruction by being adsorbed to the insoluble spleen material. When this insoluble material is filtered off most of the products of autolysis remain in the filtrate.

4. The cathepsin is then released from the insoluble spleen material by making the suspension slightly alkaline. Some inert protein still remains adsorbed to the insoluble spleen material.

5. More inert protein is removed by adsorption with aluminum hydroxide formed in the cathepsin solution by the addition first of aluminum chloride and then of sodium hydroxide. Preformed aluminum hydroxide is a much less effective adsorbent.

6. The purified cathepsin is precipitated from very dilute solution with tungstic acid. Tungstic acid precipitates most proteins in the native form provided the solution is not too acid.

7. Further evidence is given that cathepsin is not a proteinase of the papain type.

REFERENCES

Anson, M. L., 1937, *J. Gen. Physiol.*, **20**, 565.

Anson, M. L., 1938, *J. Gen. Physiol.*, **22**, 79.

Anson, M. L., 1939, *J. Gen. Physiol.*, **23**, 239.

Fruton, J. S., and Bergmann, M., 1939, *J. Biol. Chem.*, **130**, 19.

Hiller, A., and Van Slyke, D. D., 1922, *J. Biol. Chem.*, **53**, 253.

Maver, M. E., 1939, *J. Biol. Chem.*, **131**, 127.

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THE VIABILITY OF PNEUMOCOCCI IN DRIED RABBIT BLOOD

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As the literature on the life span of pneumococcus has recently been reviewed by White,[1] it is needless to recite it in detail. Working with dried sputum, Wood[2] found that pneumococcus lived for an average of 35 days in the dark, and 30 days in diffuse daylight. It has also been shown that although type specific pneumococci have a tendency to die rapidly even when protected by the blood and mucus in dried sputum, occasionally they will persist for many weeks.[3] The length of viability differed with the different types of pneumococci. The period of survival of all types studied was lengthened when the sputum was stored in the cold. There was no way of knowing, however, how many organisms, if any, were contained in each sample of sputum. In order to learn how long definitely measured quantities of pneumococci of various specific types would survive under different conditions simulating a natural environment, the following experiments were carried out.

Methods

Eighteen hour plain broth cultures of pneumococci of types I, II, and III, both virulent "smooth" cultures and avirulent "rough" R forms derived from these types, were centrifuged and the bacterial cells resuspended in normal defibrinated rabbit's blood in 1/10 of the original volume. Two-tenths cc. of the bacterial suspension was then pipetted into one series of test tubes. In another series of test tubes a one inch square of gauze had already been placed and then sterilized. The inoculated blood was dropped onto the gauze in these tubes. The blood in both series of tubes was allowed to dry at room temperature. Both series were done in triplicate. One set of tubes was placed in wire baskets and hung on the wall, another set was stored at room temperature in the dark. The average temperature of the room was 80 F. After the blood had dried, generally within 24 hours, occasionally 48 hours, the third set of tubes was stored in the icebox at a temperature of about 40 F. During the period of preservation, the tubes containing the dried material were closed only with a cotton plug.

1. The Biology of Pneumococcus, New York, The Commonwealth Fund, 1938.
2. J. Exper. Med. 7: 592, 1905.
3. J. Infect. Dis. 63: 340, 1938.

Similar experiments in which the pneumococci were frozen, dried in vacuo and stored in sealed tubes will be reported in another paper.

At monthly intervals 4 cc. of broth was added to the specimens of dried blood which had been stored in the light, in the dark, at room temperature and in the cold. If pneumococci were recovered from the virulent "smooth" cultures, a virulence test was performed by injecting 6 mice intraperitoneally with from 0.1 to .000001 dilution of the culture.

TABLE 1

Number of Months "Smooth" Pneumococci Will Remain Viable in Dried Rabbit Blood

Type of Pneumococci		Room Temperature at 80 F.		Icebox at 40 F.
		Daylight	Dark	Dark
I	A	1	1	12
	B	1	2	9
II	A	2	2	12
	B	2	2	12
III	A	5	7	13
	B	5	11	12

A—Stored on glass
B—Stored on gauze

TABLE 2

Number of Months "Rough" Pneumococci Will Remain Viable in Dried Rabbit Blood

Type of Pneumococci		Room Temperature at 80 F.		Icebox at 40 F.
		Daylight	Dark	Dark
I R	A	10	11	15
	B	10	15	9
II'R	A	3	9	9
	B	9	13	8
III R	A	0	1	7
	B	7	9	9

A—Stored on glass
B—Stored on gauze

Viability of pneumococci.—The length of time "smooth" virulent pneumococci remained viable in dried rabbits' blood under the conditions of this experiment is shown in table 1. From this it is seen that there is no significant difference between the tubes in which the blood was dried on gauze or in the bottom of the tube.

There was little, if any, difference between the tubes stored in the light or dark. Most of the type I pneumococcus cultures remained viable only a month. The type II pneumococcus cultures survived only 2 months at room temperature while the type III pneumococcus cultures lived for 5 months in daylight and 2 months longer in the dark at room temperature. In the case of all 3 types, the organisms preserved at icebox temperature remained viable for an average of 12 months. So long as the cultures remained viable, they maintained their initial type specificity and their intraperitoneal virulence was undiminished.

The length of time the R cultures derived from these type specific strains remained viable in dried rabbits' blood is shown in table 2. From this it is seen that unlike the encapsulated organisms, the "rough" variants died irregularly. Type I R lived for 10–15 months in the daylight but lasted only for 9 months on gauze in the icebox. In the case of type II R, although the cultures could not be recovered after 3 months in blood they lasted for 9 months on gauze in the light. Although the type III R did not survive even a month in the light, on gauze this strain was recovered after 7 months.

DISCUSSION

It has already been shown that pneumococci, when protected in dried pneumonic sputum, may occasionally persist for from 4 to 8 weeks. The period of survival was lengthened from 17 to 22 weeks for the same specimens when stored in the cold. But in the present experiment, when comparatively large quantities of "smooth" organism were suspended in rabbit blood and then dried, their period of survival was much shorter than when preserved in dried pneumococcus sputum. The same tendency, however, for the type I organism to die most rapidly and the type III to live longest was also noted. The effect of cold in prolonging the viability was equally as marked. But the recovery of the "rough" variants of the same types was irregular. The variants of type I remained viable longer than the type III R pneumococci. Although the dried "rough" organisms stored in the icebox lived slightly longer, the difference is not as marked as with the smooth organisms.

In the case of the dried sputum, the mucin present may have acted as a protection against autolytic enzymes of the organisms or there may have possibly been some substance in the sputum which inhibited the lytic enzymes contained in the pneumococcus cell itself. It is possible that the large number of organisms present in the dried blood liberated lytic enzymes more rapidly than the small number of organisms present in pneumonic sputum.

So little is known about the physiology of the "rough" organisms that it is difficult to explain why, under similar conditions, they should survive so much longer. In general, it has been observed, however, that the R variants undergo autolysis less readily than do the fully encapsulated cells from which they have been derived.

SUMMARY

Types I and II pneumococci preserved in dried rabbit blood survived for 1 month when stored in the light and dark at room temperature.

Under the same conditions, type III pneumococci will remain viable for 5 months.

All the three types of pneumococci will survive for about 12 months when similarly dried and stored in the cold.

The types I, II and III pneumococci which survived remained type specific and their intraperitoneal virulence was undiminished.

Rough pneumococci derived from the same types of virulent encapsulated forms live longer under the same conditions than do the smooth forms. The survival of rough organisms bears no relation to the type from which they were derived and lower temperatures only prolong their recovery a comparatively short time.

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SUSCEPTIBILITY OF MICE TO INTRANASAL INSTILLATION OF VARIOUS TYPES OF PNEUMOCOCCI

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An understanding of the epidemiology of pneumonia necessitates a knowledge of the ability of organisms to invade, survive and multiply in the tissue once they have been implanted on their surface. The reaction of various laboratory animals to organisms which have been implanted on the mucous membrane of the respiratory tract has been studied. Mice have usually been employed, since they readily succumb to infection when the organisms are inoculated directly into the tissues. A comparison of the reaction of freshly isolated strains in the same host offers an opportunity to study the variations that occur in various strains of the same and different types of organisms. In a previous paper[1] the results of exposing mice to freshly isolated strains of types I, II, III and VIII by the spray method were reported. It was demonstrated that organisms administered by this method could be found immediately after spraying in the peripheral portions of the lungs of mice.[2] As Webster[3] has shown that mice may be infected by intranasal instillation of pneumococci, it was considered advisable to try this method as a further means of testing the virulence of freshly isolated pneumococci which had clinically demonstrated their ability to invade and multiply in the human host.

Materials and Methods

Strains of pneumococci, freshly isolated from human cases of lobar pneumonia associated with types I, II, and III, were employed. Most of the cultures were isolated from the sputum or blood of patients with pneumococcus pneumonia; a few of the strains were recovered directly from spinal fluid. As soon as possible after isolation in pure culture, each strain was tested by intraperitoneal injection of 2 mice with a broth culture in amounts of .00001 and .000001 cc. If both mice died within 48 hours, the culture was classified as "virulent." If the mice died later, or if only one mouse suc-

1. Stillman, E. G. and Schulz, R. Z.: J. Infect. Dis. 65: 246, 1939.
2. Stillman, E. G.: J. Exper. Med. 38: 117, 1923.
3. J. Exper. Med. 58: 465, 1933.

cumbed, the strain was considered "slightly virulent;" if neither mouse died, the strain was considered as "non-virulent" for these animals.

At the same time, a drop of broth culture was dropped into the nose of each of 10 mice; 2 cc. syringe was used with a 23 gauge needle. Care was taken not to injure the nasal mucosa. The needle was never allowed to even touch the nose.

The virulence of a given strain was determined by the number of mice which succumbed to fatal septicemia following intranasal instillation. Since it was found in the inhalation experiments that normal mice could not be infected with pneumococci of types I and II without first being intoxicated with alcohol, the mice used in testing the invasiveness of these two types were first intoxicated by the intraperitoneal injection of alcohol. The use of alcohol is not necessary in the case of mice which were infected with type III cultures. After exposure, the mice were kept under observation for 14 days.

EXPERIMENTAL

The virulence of type I pneumococci for mice.—Fifty-nine strains of type I pneumococci were isolated from as many patients. Of the strains tested, only 2 were found to be "virulent," for mice following intraperitoneal injection, 37 were "slightly virulent" and 20 proved to be "non-virulent" as defined above.

The virulence of these strains was also tested by intranasal instillation; out of a total of 587 alcoholized mice, into the nose of which a drop of pneumococcus culture was instilled, only 29 mice died, and in only 16 instances, representing 7 strains, were pneumococci recovered from the heart's blood. Of the 7 strains which produced a fatal infection in 1 or more animals, only 1 strain caused a fatal infection in 6 out of the 10 mice exposed. From these results it is seen that the virulence of type I pneumococci as tested by the nasal route or by intraperitoneal injection is relatively slight for mice.

The virulence of type II pneumococci.—Thirty-eight cultures of type II pneumococci isolated from 37 patients suffering from pneumococcus pneumonia were tested. Of these, 32 strains were "virulent" for mice injected intraperitoneally, 4 were "slightly virulent," and only 2 were "non-virulent." Of the 362 alcoholized mice which were infected by way of the nose with cultures of these 38 strains, 145 animals died, and pneumococci were recovered from 111, or 32%. A number of these strains were highly virulent for mice, causing a fatal septicemia in 6 or 8 of the 10 mice which had been intranasally inoculated. But other strains which were "virulent" when tested by the intraperitoneal method, failed to kill mice after nasal instillation. From the result of the nasal instillation and the intraperitoneal tests it is evident that the virulence of type II pneumococci for mice is greater than that of type I.

The virulence of Type III pneumococci.—The results of the intraperitoneal virulence done on the 83 strains of type III pneumococci isolated from as many patients showed that 71 were "virulent," 8 "slightly virulent," and only 4 "non-virulent." Of the 826 normal mice into the nose of which the cultures were instilled, 235 died and from the heart blood of all of these animals, pneumococci of the homologous type were recovered. Two of the 4 strains which were "non-virulent" by the intraperitoneal injection killed 4 out of 20 mice when administered by intranasal instillation, the remaining 2 strains failed to cause the death of any of the test mice.

So it is apparent that 2 strains, which were non-virulent by intranasal instillation, were found to be virulent when the organisms were injected directly by the intraperitoneal route. From this it is seen that the virulence of a strain of pneumococcus depends somewhat on the route by which the organism is introduced into the body.

TABLE 1

Day of Death of Mice from Pneumococcus Septicemia Following Nasal Instillation

Day	1	2	3	4	5	6	7	8	9	10	11	12
Type I	0	1	4	2	1	2	2	1	3			
Type II	0	32	60	12	8	1	1	2	1		1	1
Type III	0	25	63	42	37	30	16	10	2	6	2	2
Total	0	58	127	56	46	33	19	13	6	6	3	3

Day of death following exposure.—Since under the experimental conditions the date of exposure was known, it is interesting to observe how soon after intranasal implantation the animals died of pneumococcus septicemia. The mice were kept under observation for a period of 2 weeks. The day on which the mice died following exposure is shown in table 1. From this it is seen that animals died from the second to the twelfth day. Of the deaths, 65% occurred within the first 7 days after exposure. There is no significant difference in the day of death after exposure to the different types of pneumococci used nor was there any correlation between the mortality rate of a given culture and the length of time an animal would survive.

Of the 587 alcoholized mice into the nose of which type I pneumococci were instilled, only 16, or 2%, died of pneumococcus septicemia. Of the 362 animals similarly exposed to type II pneumococci, 119, or 32%, died. Of the 826 normal mice intranasally inoculated with type III pneumococci, 235, or 28% succumbed to pneumococcus septicemia. From this it is

evident that the different types of pneumococci possess vastly different powers of invasion following intranasal instillation. As type III pneumococci will invade normal mice, it is evident that the differences in virulence of types are not dependent solely on the lowering of the host's resistance by alcohol.

DISCUSSION

Slight difference was found in the invasive powers of different strains of pneumococci which were tested on 3 breeds of mice.[4] Susceptibility of different breeds of the same species of animal to a given infectious agent has been observed by others. By using a known pedigreed strain this variation may be reduced. Variations, however, in the resistance of individual mice of the same breed are a factor which cannot be evaluated prior to the time of experimentation. The use of mice of approximately the same age and weight would eliminate, to some extent, the factor of age.

The number of organisms to which the individual animal is exposed is also a factor. Animals exposed in a chamber in which the atmosphere is filled with a spray of living organisms theoretically would have an equal chance of inhaling the same number of organisms. Differences in the rate and depth of respiration and the distance from the source of spray play a rôle in the number of organisms which are implanted on the mucous surfaces and the depth to which they are carried. By the intranasal instillation method the dosage may be varied. But a portion of the drop may be lost due to sneezing, coughing, or swallowing of the infected material. Intraperitoneal injections, however, eliminate the possibility of variation in the number of organisms against which the animal must react in order to survive.

Irrespective of the route of injection, intraperitoneal injection or intranasal instillation, type I pneumococci has the lowest incidence of mortality in mice. However, the tables compiled by White[5] show that the highest incidence of lobar pneumonia in men is associated with type I pneumococci. In infants and children, however, the type I pneumococcus is second in the order of frequency. In mice, on the other hand, a fatal infection following the implantation of type I pneumococci on the mucous membrane of the respiratory tract occurs only in animals which have been intoxicated with alcohol. It is interesting that acute and chronic alcoholism has long been considered a predisposing factor in human cases of lobar pneumonia. On the other hand, in infants and children, when

4. Personal observation.

5. The Biology of Pneumococcus, New York, The Commonwealth Fund, 1938.

alcoholism is not an important factor, the incidenee of type I pneumococcus infection is second in order of frequency.

Type II pneumococci caused a fatal infection in many mice following intranasal instillation. Organisms of this type exhibited a high degree of virulence when introduced by the intraperitoneal route.

In type III pneumococcus the proportion of intraperitoneal virulent strains and the intranasal virulent strains was approximately the same.

It has been found that preliminary intoxication with alcohol is not necessary in type III infection to reduce the resistance of the host. Type III pneumococci are able to penetrate and multiply in the tissues of normal mice. This sequence can be instigated by types I and II pneumococci only in mice in which the host resistance has been altered through alcoholic intoxication.

SUMMARY

Freshly isolated strains of type I pneumococci have a low virulence for mice when tested by both intraperitoneal injection and by intranasal instillation.

Strains of types II and III pneumococci have a higher virulence as measured by both methods.

In considering the virulence of pneumococci, it is important to consider the ability of this organism to gain entrance and to become disseminated throughout the body following implantation on mucous membrane of the respiratory tract.

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THE USE OF CALCIUM CHLORIDE IN THE TREATMENT OF CHILLS

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In a previous communication[1] it was reported that the intravenous injection of calcium chloride solution had been found to bring about prompt termination of a majority of chills which occurred after the administration of antipneumococcic serum. Additional studies have been made on the effect of this agent, not only on chills following serum administration but also on those due to other causes, namely, malarial chills, those following blood transfusions, and the intravenous injection of typhoid vaccine. The present paper deals with the results that have been obtained.

The preparation of calcium chloride used was a 10 per cent aqueous solution. The usual quantity injected was 10 cc. although as much as 20 cc. has been given. The solution should be injected very slowly as it has been found that if given too rapidly the chill manifestations, although initially relieved, may recur.

In all patients treated with calcium, the chills at the time of treatment were at least of moderate severity, characterized by tremor of the extremities, generalized spasticity of the skeletal muscles, cyanosis, chattering of the teeth, and a sensation of coldness. In the cases reported as having been completely relieved, the effect, indicated by relaxation of muscular spasm, cessation of tremor, and disappearance of symptoms, usually appeared within fifteen seconds of the beginning of the injection. Patients usually volunteered the information that they felt warm and comfortable.

Chills Following Administration of Antipneumococcic Serum.—Twenty-one patients with lobar pneumonia have been treated with calcium chloride during chills occurring after serum administration. In 13 cases there was complete relief. In the remaining 8 cases there was incomplete or no relief.

Chills Occurring in Malaria.—Three patients suffering from malaria (induced in the treatment of neurosyphilis) were available for study. In this group calcium chloride was administered on five occasions. Immediate relief of the chill was obtained in all five instances, but in two instances the injection caused nausea and had to be discontinued, after which the chill recurred.

Chill Reactions to Blood Transfusion.—Calcium chloride was administered during this type of chill on two occasions, both in the same patient. In one instance there was immediate termination of the chill, while in the second there was no observable effect. No explanation for these dissimilar results was apparent.

Chills Following Intravenous Injection of Typhoid Vaccine.—Observations were made on 2 patients who were given typhoid vaccine intravenously in the treatment of arthritis. Calcium chloride was administered during

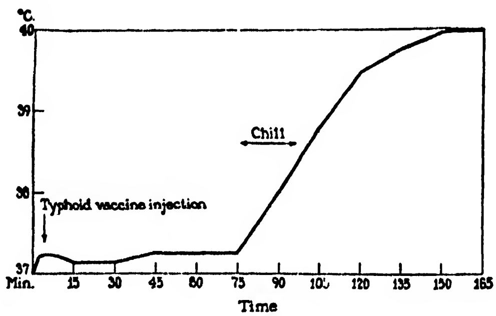

FIG. 1. Course of the rectal temperature following intravenous injection of typhoid vaccine.

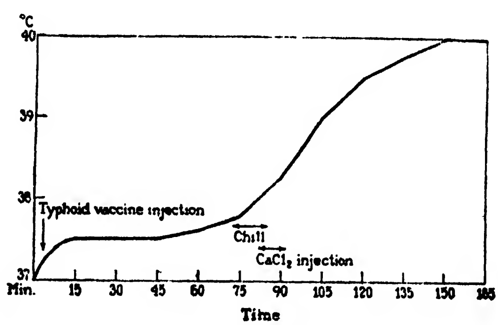

FIG. 2. Course of the rectal temperature following intravenous injection of typhoid vaccine. The chill was terminated abruptly by injection of calcium chloride.

seven chills occurring in these 2 patients. In three instances there was complete relief, in two there was marked diminution in the intensity of the chills, and in two there was no apparent effect.

Effect on Body Temperature.—Termination of the chill by calcium chloride did not appear to have any effect on the subsequent elevation of body temperature. Continuous records of the rectal temperatures of patients during chills following intravenous injection of typhoid vaccine were obtained by means of an apparatus designed by Dr. J. Murray Steele.[8] A thermocouple inserted into the rectum is connected to a galvanometer.

A beam of light is deflected by the galvanometer onto a slowly moving strip of photographic film, thus providing a continuous record of variations in the rectal temperature. Figs. 1 and 2 are diagrammatic representations of two such tracings. In both cases the chill occurred about one hour and fifteen minutes after the intravenous administration of typhoid vaccine. It will be noted that the rectal temperature began to rise at about the time of onset of the chill and continued to rise for almost one hour after the chill had ceased. No calcium chloride was administered during the period in which Fig. 1 was made. On the other hand Fig. 2 illustrates an instance in which the administration of calcium chloride brought about prompt relief of the chill. As in the other cases cited, irrespective of the causative factor, there was no significant difference in temperature response whether the chill was allowed to run its natural course or was aborted by the administration of calcium chloride.

DISCUSSION

Reports of toxic effect from the therapeutic use of calcium chloride are uncommon. It probably should not be given intravenously to patients who are receiving digitalis because calcium and digitalis have an additive effect.[3, 4, 5] Intravenous injections must be made carefully since extravasation of calcium chloride into the subcutaneous tissues may cause necrosis. The only untoward effect encountered during these studies was the occasional occurrence of nausea, which, in 3 cases was severe enough to result in vomiting. The sensation of nausea usually came on rather suddenly and occurred most frequently in the malaria patients, who were often somewhat nauseated as a consequence of the malaria itself. The result to be obtained in individual cases was not easily predictable, although in general the beneficial effect of calcium chloride was found to be less marked on the severe chills than on those of only moderate severity.

SUMMARY

Intravenous injection of calcium chloride has been found in a majority of cases to terminate chills due to various causes. Relief of the chill appeared to have no effect on the subsequent elevation of the body temperature.

REFERENCES

1. Beeson, Paul B., and Hoagland, Charles L.: Proc. Soc. Exper. Biol. & Med. **38**: 160 (1938).
2. Steele, J. Murray: Am. Heart J. **13**: 542 (1937).
3. Bower, J. O., and Mengle, H. A. K.: J. A. M. A. **106**: 1151 (1936).
4. Golden, J. S., and Brams, W. A.: Ann. Int. Med. **11**: 1084 (1938).
5. McGuigan, Robert A., and Higgins, John A.: J. Lab. & Clin. Med. **23**: 839 (1938).

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Vol. 62, No. 6, pp. 1616–1617]

THE STRUCTURE OF TRIMETHYL GLUCURONE

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(Received for publication, March 29, 1940)

Pryde and Williams[2] have prepared from glucurone a crystalline trimethyl derivative which they regard to be 1,2,4-trimethylglucurone (I). Although no evidence substantiating this structure has been presented, Marrack and Carpenter[3] also refer to the trimethylglucurone as having this structure. Such a substance on hydrolysis and oxidation might be expected to yield 2,4-dimethylsaccharic acid, which is wanted as a reference compound in this Laboratory.

When the trimethylglucurone, m. p. 129–130°, was prepared, its properties were found to resemble those of a methylfuranoside more than the methylpyranoside called for by structural formula I. On acid hydrolysis reducing sugar was liberated at the rapid rate characteristic of methylfuranosides.[4] Pryde and Williams have noted the rapid fall in rotation when trimethylglucurone is dissolved in methanol containing hydrogen chloride. It is now shown that this fall in rotation is accompanied by the formation of an isomeric trimethylglucurone, m. p. 90–91°, having a low rotation. This suggests the rapid mutarotation which Levene and Meyer[5] have observed for α- and β-methylfuranosides under similar conditions.

The high melting trimethylglucurone was hydrolyzed and oxidized to a dimethylsaccharic acid which was characterized as the crystalline diamide. When the crude dimethylsaccharic acid was esterified with cold ethereal diazomethane, no crystalline dimethylsaccharic acid esters or lactone esters were obtained; instead there was produced an unsaturated dimethyl lactone methyl ester (II) known to have its methyl groups on positions 2 and 5.[6]

(1) Present address: Boyce Thompson Institute for Plant Research, Inc., Yonkers, N. Y.

(2) J. Pryde and R. T. Williams, *Biochem. J.*, **27**, 1205 (1933).

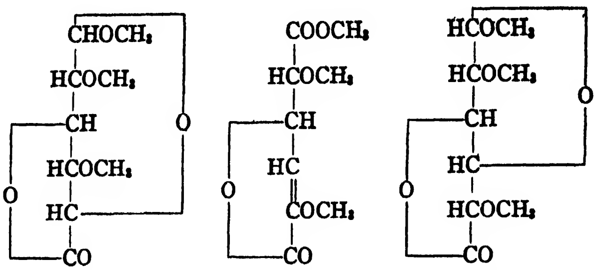
(3) J. Marrack and B. R. Carpenter, *Brit. J. Exptl. Path.*, **19**, 59 (1938).

(4) W. N. Haworth and E. L. Hirst, *J. Chem. Soc.*, 2615 (1930).

(5) P. A. Levene and G. M. Meyer, *J. Biol. Chem.*, **74**, 701 (1927).

(6) O. Th. Schmidt, H. Zeiser and H. Dippold, *Ber.*, **70**, 2402 (1937).

This finding, together with the above-mentioned properties, make it appear that the furanoside structure III, 2,5-dimethyl-α-methylglycoside of glucurone, is probably the correct structure for the trimethylglucurone of Pryde and Williams. The low melting isomer and the diamide are regarded as 2,5-dimethyl-β-methylglycoside of glucurone and 2,5-dimethylsaccharic acid diamide, respectively.



I II III

EXPERIMENTAL PART

High-Melting Trimethylglucurone.—Pryde and Williams[1] report a 25% yield of this substance following three methylations of glucurone with silver oxide and methyl iodide. By the present procedure a 50% yield was obtained after one methylation. To 5.11 g. of glucurone was added 15 cc. of acetone and 20 cc. of methyl iodide. The vigorously stirred mixture was cooled in an ice-bath during the addition of 3 g. of silver oxide. The temperature was gradually raised to 45° with the addition of 27 g. of silver oxide during nine hours. Fifteen cc. of methyl iodide and 10 cc. of acetone were added and the mixture was then refluxed at 45–48° for five hours. The product was extracted with chloroform and crystallized from acetone by the addition of ether, a second and third crop being obtained from the mother liquors. After recrystallization 2.71 g. of 2,5-dimethyl-α-methylglycoside of glucurone, m. p. 129–130°, was obtained; sp. rot. (D-line) (24°) 151° (*c*, 0.4 in $CHCl_3$).

Low-Melting Trimethylglucurone.—Trimethylglucurone, m. p. 129–130°, (372.6 mg.) was dissolved at 23° in 15 cc. of methanol containing 3.6% dry hydrogen chloride. After seventy minutes the rotation (1-dm. tube) became constant at −0.03°. The solution was evaporated to dryness *in vacuo* and, when the residue was redissolved in methanol and again taken to dryness, crystals formed in the distillation flask. Recrystallization from methanol and ether gave 270 mg. of 2,5-dimethyl-β-methylglycoside of glucurone, m. p. 90–1°, sp. rot. (D-line) (21°) 2.0° (*c*, 1.0 in H_2O); (24°) −2.3° (*c*, 0.9 in $CHCl_3$).

Anal. Calcd. for $C_9H_{14}O_6$: C, 49.53; H, 6.42; CH_3O, 42.6. Found: C, 49.24; H, 7.15; CH_3O, 42.85.

Kinetics of Hydrolysis.—Trimethylglucurone (94.6 mg.) m. p. 129–130° was refluxed in 50 cc. of 0.05 *N* hydrochloric acid and the reducing sugars were determined at intervals by the modified Hagedorn-Jensen procedure.[7] The results are given in cc. of 0.01 *N*

(7) C. S. Hanes, *Biochem. J.*, **23**, 99 (1929).

thiosulfate required by 1 cc. of hydrolysate after refluxing for a stated length of time: 0 min., 0.11 cc.; 5 min., 1.76 cc.; 15 min., 3.91 cc.; 30 min., 4.65 cc.; 60 min., 4.49 cc. To minimize the effect of the gradual decomposition of reducing sugar a curve was drawn through the points and the velocity constant was calculated by the method of Guggenheim[8] for the first twenty minutes of the hydrolysis. The value was found to be $k = 1380 \times 10^{-5}$ min.$^{-1}$ for $N/100$ HCl at 100°, which is definitely within the range exhibited by most furanosides. When recalculated on the basis of natural logarithms the values found by Haworth and Hirst[4] for furanosides in 0.01 N acid at 100° range from 600×10^{-5} to $11{,}000 \times 10^{-5}$.

2,5-Dimethylsaccharic Acid Diamide.—Trimethylglucurone (100 mg.), m. p. 129–130°, was heated for four hours at 80–85° in 4 cc. of dilute nitric acid, sp. gr. 1.2. Nitric acid was removed by evaporating *in vacuo*, adding water and repeatedly evaporating to dryness *in vacuo*. The residue was then esterified by heating for three hours at 75° in a sealed tube with 4 cc. of methanol containing 2% dry hydrogen chloride. The hydrogen chloride was removed with silver carbonate. Attempts to obtain crystals from this ester both before and after vacuum distillation were unsuccessful. The sirup was dissolved in 2 cc. of methanol, cooled to 0°, and treated with ammonia gas. Crystals separated, and more were obtained by the addition of ether to the mother liquors; 43 mg. of crude material yielded 11 mg. of pure diamide, m. p. 169–170°, after three recrystallizations from ethanol.

Anal. Calcd. for $C_8H_{16}O_6N_2$: C, 40.68; H, 6.78; N, 11.88; CH_3O, 26.3. Found: C, 40.62; H, 6.77; N, 11.70; CH_3O, 26.46.

Unsaturated Saccharolactone Methyl Ester II.—Two hundred mg. of high-melting trimethylglucurone was hydrolyzed and oxidized with nitric acid as in the preparation of the diamide. The saccharic acid after removal of nitric acid and water was treated with 15 cc. of an 0.5 molar solution of diazomethane in ether, and allowed to stand at 0° for three days. The solution was filtered from a small flocculent precipitate, and concentrated to 3 cc. Forty mg. of crystals separated, which on recrystallization from acetone–ether melted at 85–86.5°. Mixed with the unsaturated lactone ester, m. p. 87–88°, prepared from saccharolactone methyl ester[9] the melting point was 86–87°.

SUMMARY

Evidence is presented which indicates that the trimethylglucurone prepared by Pryde and Williams is the 2,5-dimethyl-α-methylglycoside of glucurone.

(8) E. A. Guggenheim, *Phil. Mag.*, [7] **2**, 538 (1926).
(9) R. E. Reeves, This Journal, **61**, 664 (1939).

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STUDIES ON ANTIBACTERIAL IMMUNITY INDUCED BY ARTIFICIAL ANTIGENS

II. IMMUNITY TO EXPERIMENTAL PNEUMOCOCCAL INFECTION WITH ANTIGENS CONTAINING SACCHARIDES OF SYNTHETIC ORIGIN

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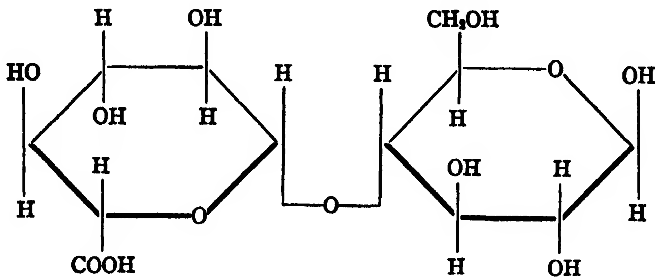
(Received for publication, April 22, 1940)

The functional rôle of bacterial polysaccharides in immunity has been amply demonstrated in studies from this and other laboratories. Although knowledge of the precise chemical constitution of the bacterial polysaccharides is still meager, it has been possible in some measure to correlate their specific immunological properties with differences in chemical structure. Several years ago we demonstrated that type specific antibacterial immunity could be evoked in rabbits with an artificial antigen containing the azobenzyl ether of the capsular polysaccharide of Type III Pneumococcus (1). More recently it has been shown that an antigen containing the azobenzyl glycoside of cellobiuronic acid, the pattern unit of the Type III pneumococcus polysaccharide, evokes in rabbits antibodies which agglutinate Type III pneumococci and confers passive immunity on mice against infection not only with Type III but with Type II and VIII organisms as well (2).

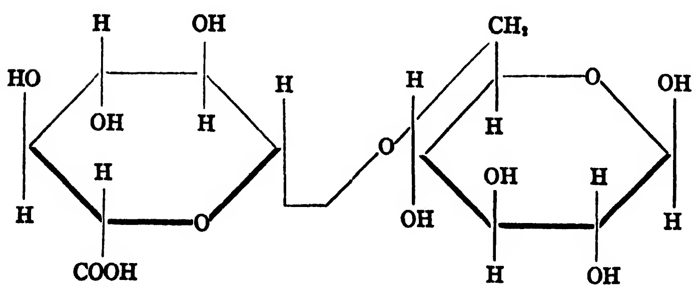
Cellobiuronic acid (4-β-glucuronosidoglucose) is an aldobionic acid constituted from a molecule of glucose linked in β-glucuronosidic union with a molecule of glucuronic acid on the fourth carbon atom of the hexose (3). Whether this exact molecular configuration is essential for eliciting pneumococcal antibodies in experimental animals is the subject of the present communication. A second antigen has therefore been prepared, one containing an isomeric aldobionic acid, gentiobiuronic acid (6-β-glucuronosidoglucose) (4). The immunological properties of the antigen containing the synthetic saccharide have been compared and correlated with those of the antigen containing cellobiuronic acid, the aldobionic acid derived from natural sources. Cellobiuronic and gentiobiuronic acids are isomers differing in the position but not the configuration of the β-glucuronosidic union as can be seen from Figs. 1 and 2.

Any differences in the immunological properties of the antigens containing the two aldobionic acids can be directly correlated with differences in their chemical constitution since the latter is known. For purposes of comparison there have been included in this investigation a study of the immunological properties of antigens containing the azobenzyl glycosides of glucuronic and galacturonic acids and the disaccharide gentiobiose. The structural relationship of these saccharides has been discussed in earlier



Cellobiuronic acid
(4-β-glucuronosidoglucose)

FIG. 1



Gentiobiuronic acid
(6-β-glucuronosidoglucose)

FIG. 2

communications. That the chemical structure of these derivatives is the sole factor determining the immunological properties of antigens in which the saccharides are incorporated will be seen from the following account.

EXPERIMENTAL

Chemical

Heptaacetylgentiobiuronic Acid Methyl Ester.—This derivative was prepared by condensing 1,2,3,4 tetracetyl glucose (5) with acetobromoglucuronic acid methyl

ester (6). By employing the method recommended by Reynolds and Evans (7) for the general synthesis of glycosides, yields of 60 per cent of the acetylated aldobionic acid methyl ester were obtained.

Acetobromogentiobiuronic Acid Methyl Ester.—14.3 gm. of heptaacetylgentiobiuronic acid methyl ester were dissolved in 60 cc. of dry chloroform. 60 cc. of glacial acetic acid saturated with hydrobromic acid were added. After 30 minutes the mixture was poured into 800 cc. of ice and water. The mixture was extracted with 250 cc. of alcohol-free chloroform and washed three times with ice water. After drying the chloroform solution with sodium sulfate, the solvent was evaporated *in vacuo*. The oily residue was dissolved in anhydrous ether whereupon crystals of acetobromogentiobiuronic acid methyl ester separated. 4.0 gm. were recovered. The derivative was recrystallized several times by dissolving in warm chloroform (40 cc.) and adding an equal volume of ether. The compound crystallized as rosettes of needles melting with decomposition at 192° (uncorrected).

Rotation.—$[\alpha]_D^{24°} = +99.2°$ in $CHCl_3$ (C = 0.8 per cent).
Analysis.—$C_{25}H_{33}O_{17}Br$. Calculated. Br 11.7.
Found. Br 11.9.

β p-Nitrobenzylglycoside of Hexaacetylgentiobiuronic Acid Methyl Ester.—1.5 gm. of *p*-nitrobenzyl alcohol were dissolved in 10 cc. of dry chloroform. 1.5 gm. of silver oxide were added and 10 gm. of Drierite previously heated for 2 hours at 240°. The mixture was stirred 1 hour in an apparatus bearing a mercury sealed stirrer. 0.32 gm. of iodine were then added and a solution of 4.3 gm. of acetobromogentiobiuronic acid methyl ester dissolved in 35 cc. of dry alcohol-free chloroform was added from a dropping funnel over a period of 1 hour. After stirring overnight, the mixture was filtered and the chloroform evaporated from the filtrate *in vacuo*. The resulting syrup was dissolved in ethyl alcohol, whereupon the glycoside crystallized from the solution. 4.3 gm. of pure glycoside were recovered; the derivative crystallizes as needles melting at 113–115° (uncorrected).

Rotation.—$[\alpha]_D^{24°} = -42.2°$ in $CHCl_3$ (C = 1.1 per cent).
Analysis.—$C_{30}H_{36}O_{18}N$ ($COOCH_3$).
Calculated. C 50.7, H 5.2, CH_3O 4.1, N 1.85.
Found. C 50.7, H 5.3, CH_3O 3.8, N 1.80.

β p-Nitrobenzylglycoside of Gentiobiuronic Acid Methyl Ester.—3.15 gm. of the acetylated glycoside were suspended in 100 cc. of cold anhydrous methyl alcohol and 1 cc. of N/1 barium methylate (8) was added. The mixture was shaken 1 hour at room temperature and then allowed to stand for 2 hours more. 1 cc. of N/1 H_2SO_4 was added, the precipitated barium sulfate removed by centrifugation, and the solution of the deacetylated glycoside evaporated *in vacuo* to 10 cc. On standing at 0° the solution deposited crystals of the *p*-nitrobenzylglycoside of gentiobiuronic acid methyl ester. 2.0 gm. were recovered. The glycoside was recrystallized several times from methyl alcohol. The substance was obtained as fine silky needles melting at 107–110° (uncorrected).

Rotation.—$[\alpha]_D^{22°} = -58.3°$ in CH_3OH (C = 0.9 per cent).
Analysis.—$C_{20}H_{27}O_{14}N$. Calculated. OCH_3 6.2, N 2.8.
Found. OCH_3 6.1, N 2.4.

Barium Salt of p-Aminobenzylglycoside of Gentiobiuronic Acid.—2.2 gm. of the β *p*-nitrobenzylglycoside of gentiobiuronic acid methyl ester were dissolved in 100 cc. of methyl alcohol and reduced catalytically with hydrogen using 50 mg. of platinum oxide as catalyst (9). The derivative utilized exactly the theoretical quantity of hydrogen. The solution was then filtered and the solvent evaporated *in vacuo*. The aminobenzylglycoside of gentiobiuronic acid methyl ester was now dissolved in 50 cc. of ethyl alcohol and one equivalent of 0.4 N barium hydroxide was slowly added from a burette. The solution was treated with Norite, filtered, and concentrated to a pale yellow oil. The latter was slowly poured into a liter of chilled acetone. The barium salt of the gentiobiuronide separated as a faintly yellow amorphous precipitate. The salt was collected by filtration; 2.18 gm. were recovered.

Rotation.—$[\alpha]_D^{24°}$ = −57.1° in H_2O (C = 0.5 per cent).
Analysis.—$C_{19}H_{26}O_{12}N\frac{1}{2}Ba$. Calculated. N 2.7, Ba 13.0.
Found. N 2.4, Ba 12.4.

Heptaacetyl β p-Nitrobenzylgentiobioside.—This derivative was prepared by condensing 1.67 gm. of acetobromogentiobiose (10) with 5.8 gm. of *p*-nitrobenzyl alcohol and 5.7 gm. of silver oxide according to the technique of Reynolds and Evans (7). The glycoside was obtained from the reaction mixture as described for the corresponding glycoside of gentiobiuronic acid methyl ester. 6.6 gm. of pure glycoside were recovered by repeated recrystallization of the reaction product from methyl alcohol. The derivative was obtained as glistening needles melting at 171–172° (uncorrected).

Rotation.—$[\alpha]_D^{25°}$ = −38.5° in $CHCl_3$ (C = 0.7 per cent).
Analysis.—$C_{33}H_{41}O_{20}N$. Calculated. C 51.4, H 5.4.
Found. C 51.3, H 5.4.

β p-Nitrobenzylgentiobioside.—5.0 gm. of the heptaacetyl β *p*-nitrobenzylgentiobioside were suspended in 100 cc. of methyl alcohol and deacetylated with barium methylate exactly as was the corresponding glycoside of gentiobiuronic acid; 2.8 gm. of glycoside were recovered from the mother liquors. The derivative crystallizes in rosettes of needles melting at 120° (uncorrected) preceded by softening at 113°.

Rotation.—$[\alpha]_D^{25°}$ = −46.8° in H_2O (C = 1.2 per cent).
Analysis.—$C_{19}H_{27}O_{13}N$. Calculated. C 47.8, H 5.6, N 2.9.
Found. C 47.9, H 5.7, N 2.6.

β p-Aminobenzylgentiobioside.—The β *p*-nitrobenzylgentiobioside was reduced catalytically according to the directions outlined for the preparation of the corresponding glycoside of gentiobiuronic acid methyl ester. In carrying out the reduction the theoretical quantity of hydrogen was utilized. It was not possible to obtain the glycoside in a crystalline state. The aminobenzylglycoside has been secured only as a syrup, having the correct analysis.

Rotation.—$[\alpha]_D$ = −49.7° in H_2O (C = 1 per cent).
Analysis.—$C_{19}H_{29}O_{11}N$. Calculated. N 3.13.
Found. N 3.08.

β p-Aminobenzylglycosides of Cellobiuronic, Glucuronic, and Galacturonic Acids.—These derivatives were prepared by methods previously described (2, 11).

Immunological

Methods.—The immunizing and test antigens containing the azo derivatives of the *p*-aminobenzylglycosides of cellobiuronic, gentiobiuronic, glucuronic, and galacturonic acids and of gentiobiose were prepared by methods previously outlined. The method of intravenous injection of rabbits as well as the technique of passive immunity tests in mice were the same as those used in former studies. Precipitin reactions and specific inhibition tests were conducted in the usual manner. For the sake of brevity, immunizing antigens will be referred to in the tables as CA-globulin (cellobiuronic acid-globulin), GeA-globulin (gentiobiuronic acid-globulin), and GaA-globulin (galacturonic acid-globulin). Test antigens prepared by combining the various glycosides with chicken serum are referred to as CA–chick, etc. The *p*-aminobenzylglycosides used as

TABLE I

Homologous and Heterologous Precipitin Reactions of Cellobiuronic Acid, Gentiobiuronic Acid, and Gentiobiose Antisera

Antiserum prepared by immunization with	Test antigen used	Final dilution of test antigen		
		1:10,000	1:25,000	1:50,000
CA-globulin	CA—chick	+++	++	++
	GeA "	++	++	+±
	Ge "	0	0	0
GeA-globulin	CA "	+±	++	0
	GeA "	+++	+++	+++
	Ge "	++±	++±	++±
Ge-globulin	CA "	0	0	0
	GeA "	+±	+±	+±
	Ge "	+++	+++	+++

inhibiting agents are referred to as CA, GeA, GlA, GaA, and Ge for the cellobiuronide, gentiobiuronide, glucuronide, galacturonide, and gentiobioside respectively.

Precipitin Reactions

Antisera of Rabbits Immunized with Antigens Containing the Azobenzyl Glycosides.—The two isomeric aldobionic acids cellobiuronic and gentiobiuronic acids are constituted from molecules of glucose and glucuronic acid linked in β-glucuronosidic union on the fourth and sixth carbon atom of the hexose respectively. The disaccharide gentiobiose differs from the corresponding aldobionic acid only in the nature of the grouping occupying the twelfth position. In the disaccharide this grouping is a primary alcohol (CH_2OH) and in the uronic acid a carboxyl group. The sera of rabbits immunized with antigens containing these three saccharides show marked precipitation with the homologous test antigens as seen in Table I. The

cellobiuronic acid antiserum cross reacts with the test antigen containing the isomeric aldobionic acid, but not with that containing the disaccharide

TABLE II

Inhibition of Precipitins in Cellobiuronic Acid Antiserum by Homologous and Heterologous Glycosides

Inhibiting glycoside	Cellobiuronic acid antiserum	
	Test antigen 1:10,000	
	CA—chick	GeA—chick
CA	0	0
GeA	+++	0
Ge	+++	+++
None	++++	+++

TABLE III

Inhibition of Precipitins in Gentiobiose Antiserum by Homologous and Heterologous Glycosides

Inhibiting glycoside	Gentiobiose antiserum	
	Test antigen 1:10,000	
	Ge—chick	GeA—chick
Ge	0	0
GeA	+++	0
CA	+++	++
None	+++±	++

TABLE IV

Inhibition of Precipitins in Gentiobiuronic Acid Antiserum by Homologous and Heterologous Glycosides

Inhibiting glycoside	Gentiobiuronic acid antiserum		
	Test antigen 1:10,000		
	GeA—chick	Ge—chick	CA—chick
GeA	0	0	0
Ge	+++	0	++±
CA	+++	++	0
None	+++	++±	++±

gentiobiose. Likewise the gentiobiuronic acid serum cross reacts with the cellobiuronic acid antigen. This cross reaction can be attributed only to

the common glucuronic acid constituent. Gentiobiuronic acid antiserum on the other hand cross reacts with the gentiobiose test antigen and *vice versa*. These cross reactions can in all probability be directly attributed to the close constitutional similarities of the two saccharides in question. It is of interest to observe that the antiserum evoked by the antigen containing the disaccharide gentiobiose does not cross react with the cellobiuronic acid test antigen. Although these two saccharides have a glucose molecule in common, it is apparent that this common constituent is not reflected in the immune sera either to the cellobiuronic acid or gentiobiose antigen. That the antibodies evoked by each antigen are indeed specific and that the cross reactions are quite secondary is evident from the results of the

TABLE V

Precipitin Reaction of Gentiobiose and Gentiobiuronic Acid Antigens in Antipneumococcal Horse Sera

Antipneumococcus horse serum	Test antigen used	Final dilution of test antigen			
		1:10,000	1:50,000	1:250,000	1:1,000,000
II	Ge—chick	+±	±	0	0
	GeA "	++	+±	+	±
III	Ge "	+	±	0	0
	GeA "	+++	++	+±	+
V	Ge "	+	0	0	0
	GeA "	++++	+++	++	+
VIII	Ge "	++±	+±	±	0
	GeA "	+++	+±	+	±

specific inhibition tests given in Tables II, III, and IV. Here it can be seen that in all instances the homologous reaction is inhibited only by the homologous glycoside and in no instance by heterologous glycosides. The cross reactions, as might be expected, are inhibited in all cases by the homologous glycoside and by the glycoside common to each test antigen.

Precipitin Reactions of Antipneumococcal Sera with Test Antigens Containing the Glycosides.—In previous studies we have shown that artificial antigens containing cellobiuronic and glucuronic acids react in antipneumococcal horse sera Types II, III, and VIII in dilutions as high as one part in a million of the test antigen. It has now been found that gentiobiuronic acid test antigen likewise reacts in these antisera and in Type V antipneumococcal horse serum as well (Table V). The precipitation of these

uronic acid antigens in antipneumococcal horse sera has been attributed to the reaction of the antigen with uronic acid antibodies evoked in the horse by the type specific bacterial antigen which likewise contains uronic acids. The precipitation of the gentiobiuronic acid antigen in Type V antiserum would be difficult to understand on the basis of the work of Brown (12) who reports that the Type V polysaccharide contains no uronic acid. In this laboratory, however, we have recently isolated the Type V polysaccharide and have found that after acid hydrolysis it gives a distinct color

TABLE VI

Inhibition of Precipitin Reactions of Cellobiuronic and Gentiobiuronic Acid Antigens in Antipneumococcus Horse Sera

Antipneumococcus horse serum Type	Inhibiting glycoside M/10	Test antigens (1:10,000)	
		CA—chick	GeA—chick
II	CA	0	0
	GeA	0	0
	GlA	0	0
	None	+++	+++
III	Ca	0	0
	GeA	++++	0
	GlA	++++	0
	None	++++	+++
V	CA	0	0
	GeA	0	0
	GlA	0	0
	None	+++	+++
VIII	CA	0	0
	GeA	++++	0
	GlA	++++	0
	None	++++	+++

test for uronic acids with naphthoresorcinol. That the precipitation of gentiobiuronic acid test antigen in the antipneumococcal sera is a function of the uronic acid constituent is apparent from the results presented in Table V, for here it is seen that the corresponding antigen containing the disaccharide gentiobiose shows little or no precipitation in these types of antipneumococcal sera, save that of Type VIII. The reason for the precipitability of the disaccharide antigen in this type of antipneumococcal serum cannot be given until a more comprehensive understanding of the chemical makeup of the Type VIII polysaccharide is had.

The results of the inhibition of the precipitin reactions of gentiobiuronic and cellobiuronic acid antigens in antipneumococcal horse sera of various types is given in Table VI. It will be observed that the precipitation of both aldobionic acid antigens in Type II and V antipneumococcal sera is in all instances inhibited indiscriminately by the *p*-aminobenzylglycosides of glucuronic, gentiobiuronic, or cellobiuronic acids. Furthermore, the precipitation of the gentiobiuronic acid antigen in Type III and VIII antisera is also inhibited by all of the glycosides, but the reaction of cellobiuronic acid antigen in these same sera is inhibited only by the homologous glycoside.

Now it has been demonstrated (13) that cellobiuronic acid is a constituent of the Type III and VIII pneumococcus polysaccharides. The results of these specific inhibition tests indicate, therefore, that the precipitation of the cellobiuronic acid antigen in these two types of antipneumococcal sera is of a more specific nature than is the precipitation of the gentiobiuronic acid antigen. In a sense, the reaction of the cellobiuronic acid antigen in Type III and VIII antisera may be regarded as type specific. The precipitation of the gentiobiuronic acid antigen appears to be non-specific. The reaction occurs only by virtue of the glucuronic acid constituent, since it is inhibited by any glycoside containing glucuronic acid. The precipitation of the two aldobionic acid antigens in Type II and V antisera may likewise be considered as non-specific for here again both reactions are inhibited indiscriminately by all the uronides. From the results of these tests it might indeed be inferred that neither cellobiuronic nor gentiobiuronic acids are constituents of the Type II and V pneumococcus polysaccharides. This inference must await further experimental proof before it is possible to say that the aldobionic acid constituents of bacterial polysaccharides can be determined by serological as well as chemical technique.

Neufeld Quellung Reactions.—In the previous communication (2) it was shown that the sera of rabbits immunized to the artificial antigen containing cellobiuronic acid showed a typical swelling of the capsule of young actively growing Type III pneumococci when the organisms and serum were mixed. In the present study we have observed that the sera of rabbits immunized to the isomeric gentiobiuronic acid cause no detectable swelling of the capsule of Type II or III pneumococci. This point will be discussed further in the section dealing with the protective action of these antisera.

Agglutinins.—The sera of rabbits injected with the two aldobionic acid antigens were tested for agglutinins with heat-killed suspensions of Type II and III pneumococci. The results of these experiments are given in Table VII. Here it can be seen that the sera of rabbits immunized to the

cellobiuronic acid antigen agglutinate Type III pneumococci (as previously found), but not Type II organisms whereas the gentiobiuronic acid antiserum fails to show any demonstrable agglutinating activity with pneumococci of either type.

TABLE VII

Agglutination of Type II and III Pneumococci in Cellobiuronic and Gentiobiuronic Acid Antisera

Antiserum prepared by immunization with	Pneumococcus Types	Final dilution of serum				
		1:10	1:20	1:40	1:80	1:160
Cellobiuronic acid globulin	II	0	0	0	0	0
	III	++	+++	+++	++	+
Gentiobiuronic acid globulin	II	0	0	0	0	0
	III	0	0	0	0	0

TABLE VIII

Protective Action of Gentiobiuronic Acid Antiserum against Pneumococcal Infection in Mice

Amount of culture	Pneumococcus			
	Type II	Type III	Type V	Type VIII
cc.				
10^{-3}	S D 108	— —	— —	
10^{-4}	S S	— —	— —	
10^{-5}	S S	D 40 D 40	D 40 D 40	D 24 D 24
10^{-6}	S S	D 40 D 42	D 40 D 40	D 24 D 36
Virulence controls (no serum)				
10^{-6}	D 48	D 40	D 28	D 24
10^{-7}	D 48	D 40	D 40	D 24
10^{-8}	D 48	D 48	D 48	D 48

In this and the following tables S indicates survived; D indicates death, the numerals representing the hours before death of the animal occurred.

Protective Antibodies. A. Gentiobiuronic Acid Antiserum.—It has been previously shown (2) that the serum of rabbits immunized to the artificial antigen containing cellobiuronic acid confers passive immunity on mice against infection with Type II, III, and VIII pneumococci. In the present study protection tests have been done in mice infected with Type II, III, V, and VIII pneumococci using gentiobiuronic acid antiserum. The results of these tests are given in Table VIII.

From the results given in Table VIII it is apparent that the sera of rabbits injected with the gentiobiuronic acid antigen, unlike those immunized with the cellobiuronic acid antigen (2) fail to protect mice against infection with Type III, V, or VIII pneumococci. However, the gentiobiuronic acid antiserum does confer passive immunity on mice against infection with as much as 100,000 minimal lethal doses of Type II organisms, although the antiserum apparently neither agglutinates nor causes swelling of the capsule of this particular type of organism. It is possible to demonstrate that the protective antibodies are specifically absorbed by encapsulated Type II pneumococci but not by the unencapsulated R variant derived therefrom (Table IX).

TABLE IX

Protective Action of Gentiobiuronic Acid Antiserum before and after Absorption with Type II S and R Pneumococci

Pneumococcus Type II	Gentiobiuronic acid antiserum		
	Unabsorbed	Absorbed with Type II S pneumococci	Absorbed with R organisms derived from Type II Pneumococcus
cc.			
10^{-4}	S S	D 24 D 24	S D 108
10^{-5}	S S	D 24 D 24	S S
10^{-6}	S S	D 48 D 48	S S
Virulence controls (no serum)			
10^{-6}	D 48		D 48
10^{-7}	D 24		D 48
10^{-8}	D 48		D 48

According to the older classification of pneumococci, the present Type V was considered a subgroup of Type II, for it was known that Type II antiserum protected mice against infection with atypical II *a* (Type V) organisms. Although the gentiobiuronic acid antigen precipitates in high dilution in Type V antipneumococcal horse serum (Table V) the antiserum to the uronic acid fails to protect mice against infection with Type V organisms. The reason for this is at present not clear.

B. Gentiobiose Antiserum.—In the previous study of this series (2) it was demonstrated that an antiserum evoked by an antigen containing the disaccharide cellobiose failed to protect mice against infection with Type II, III, and VIII pneumococci although the corresponding cellobiuronic acid antiserum did. Similarly we have now found that the antibodies evoked by an antigen containing the azobenzyl glycoside of gentiobiose

(Table X) fail to protect mice against infection with Type II pneumococci. This fact emphasizes once again the importance of the uronic acid structure in determining the nature of the immunological response in the host.

C. Glucuronic and Galacturonic Acids.—The fact that antisera evoked by antigens containing the isomeric cellobiuronic and gentiobiuronic acids both confer immunity on mice against infection with Type II pneumococci and at the same time show highly specific differences in protecting mice against Type III pneumococcal infection seems paradoxical. However, cellobiuronic and gentiobiuronic acids contain a common constituent, namely glucuronic acid, which might be capable of evoking Type II antibacterial immune bodies in rabbits. Consequently the sera of rabbits immunized to antigens containing the simple hexose uronic acids glucuronic and galacturonic acids were tested for their ability to protect mice against

TABLE X

Protective Action of Gentiobiose Antiserum against Type II Pneumococcal Infection in Mice

Pneumococcus Type II	Gentiobiose antiserum
cc.	
10^{-5}	D 24 D 48
10^{-6}	D 72 D 72
10^{-7}	D 72 D 48
Virulence controls (no serum)	
10^{-6}	D 48
10^{-7}	D 24
10^{-8}	S

infection with Type II pneumococci and at the same time an immune serum to an antigen containing the hexose glucose was also tested. It will be recalled that the antisera evoked in rabbits by antigens containing glucuronic and galacturonic acids show no serological crossings (11). It will be recalled, furthermore, that glucuronic and galacturonic acids differ only in the configuration of the fourth asymmetric carbon atom, yet this structural difference endows each antigen with a clearly defined serological specificity. Glucuronic and galacturonic acid antisera exhibit sharply defined specificities not only in their serological characteristics but in their ability to confer passive immunity on mice to Type II pneumococcal infection. The antisera to galacturonic acid is quite ineffectual in conferring passive immunity against Type II pneumococcal infection. The antiserum to the antigen containing the synthetic glucuronide on the other hand pro-

tects mice against infection with 100,000 minimal lethal doses ot Type II pneumococci, but not against Type III organisms (Tables XI and XII). An antiserum elicited by an antigen containing glucose, on the other hand,

TABLE XI

Protective Action of Glucose, Glucuronic Acid, and Galacturonic Acid Antisera against Type II Pneumococcal Infection in Mice

Pneumococcus Type II	Antiserum tested		
	Glucose-globulin	Glucuronic acid-globulin	Galacturonic acid-globulin
cc.			
10^{-3}	— — —	D 72 D 66 S	— —
10^{-4}	— — —	S S S	D 24 D 24
10^{-5}	D 24 D 24 D 24	S S S	D 24 D 24
10^{-6}	D 24 D 44 D 44	S S S	D 24 D 24
10^{-7}	D 14 D 66 S	— — —	— —
Virulence controls (no serum)			
10^{-6}	D 18 D 44	D 24 D 24	D 24
10^{-7}	D 24 D 24	D 48 D 48	D 24
10^{-8}	D 24 D 24	D 48 D 48	D 48

TABLE XII

Protective Action of Glucuronic Acid Antiserum before and after Absorption with Type II S and R Pneumococci

Pneumococcus Type II	Glucuronic acid antiserum		
	Unabsorbed	Absorbed with Type II S pneumococci	Absorbed with R organisms derived from Type II Pneumococcus
cc.			
10^{-4}	S S	D 24 D 24	S D 48
10^{-5}	S S	D 24 D 24	S S
10^{-6}	S S	D 24 D 24	S S
Virulence controls (no serum)			
10^{-6}		D 24 D 48	
10^{-7}		D 24 D 24	
10^{-8}		D 24 D 48	

contains no protective antibodies against Type II infection. By means of absorption tests (Table XII) it has also been possible to show that the protective antibodies in glucuronic acid antiserum are specifically absorbed

by encapsulated Type II pneumococci but not by the corresponding unencapsulated R strain. From the results of these experiments it seems justifiable to conclude that the protective action of cellobiuronic, gentiobiuronic, and glucuronic acid antisera against Type II pneumococcal infection in mice is in each instance attributable to antibodies evoked by the glucuronic acid constituent.

DISCUSSION

In view of increasing evidence accumulated during the past few years there remains little doubt that the specificity of the various pneumococcal types may be directly attributed to differences in chemical nature of their capsular polysaccharides. Our knowledge of the precise chemical structure of the specific carbohydrates of this important group has unfortunately remained obscure ever since the time of their discovery some seventeen years ago. The technical and economic difficulties encountered in procuring sufficient material for investigation by the classical methods of organic chemistry have in themselves been a factor in deterring the pursuit of this problem. Most of the efforts in this and other laboratories have been confined to an investigation of the physical properties of the polysaccharides, to the identification of the sugars from which they are constituted, and to a study of their immunological characteristics. The fact remains, however, that an understanding of the specificities of the various types of pneumococci will be had only after the structures of their capsular polysaccharides have been fully elucidated. The problem has become one strictly chemical in nature and its magnitude is emphasized by the recent contribution of Brown (12) who has now isolated and in part characterized the specific substances from all of the known pneumococcal types hitherto unstudied.

The study of the factors underlying the specificity of immunologically active polysaccharides has been greatly facilitated by the use of the method so brilliantly conceived and developed by Landsteiner and his collaborators in their immunological investigations of azoprotein antigens (14). Many of our efforts have been devoted to an investigation of the immunological properties of artificially conjoined carbohydrate-proteins containing saccharides of known structure. The fertility of this field remained somewhat limited until it was discovered that artificial antigens containing simple hexose uronic acids acquired some of the serological characteristics of the pneumococcal polysaccharides themselves. Knowledge of the close relationship of these artificial antigens to the bacterial polysaccharides has now stimulated efforts to investigate further the specific immunological properties of uronic acids and has led to experiments in which attempts have been made to incite in experimental animals antibacterial immunity with

artificially compounded substances. Earlier efforts to evoke in rabbits immunity to Type I, III, and VIII pneumococci with antigens containing glucuronic and galacturonic acids were unsuccessful. Recently however it was found that when an antigen is employed containing the azobenzyl glycoside of cellobiuronic acid, the artificially prepared complex gives rise in rabbits to antibodies which confer passive immunity on mice to infection with Type II, III, and VIII pneumococci.

The uronic acid constituent of the Type II carbohydrate is still unknown and it is therefore not easy to explain the protective action of cellobiuronic acid antiserum on mice infected with this type of microorganism. Cellobiuronic acid has however been identified as a constituent of the Type III and VIII polysaccharides and the immunological properties exhibited by the corresponding uronic acid antiserum are therefore quite understandable. Whether the exact molecular structure possessed by cellobiuronic acid is the essential factor which endows this substance with these important immunological properties has been the subject of the present investigation.

From the results of experiments in which an antigen containing an isomer of cellobiuronic acid namely gentiobiuronic acid has been used it is evident that an alteration in position without change in configuration of the glucuronosidic linkage endows the new antigen with distinctly different immunological properties. The antigen containing the synthetic gentiobiuronic acid is incapable of inciting in rabbits antibodies which agglutinate Type III pneumococci or confer passive immunity on mice against infection with either Type III or VIII organisms. It is apparent, therefore, that the particular structure of cellobiuronic acid determines not only the immunological specificity of the aldobionic acid but in part that of the parent polysaccharide of which it forms a structural unit. The same principle is applicable in explaining the protective action of cellobiuronic acid antiserum against Type VIII pneumococcal infections although in this instance the complete pattern unit is not as yet fully known.

Without knowledge of the uronic acid constituent of the Type II pneumococcus polysaccharide it is difficult to understand the protective action of both cellobiuronic and gentiobiuronic acid antisera against infection with this type of microorganism. The explanation becomes clearer however since it is now known that glucuronic acid antiserum also has this property. The protective antibodies in all three antisera are obviously those directed toward the common glucuronic acid constituent. These observations and the fact that the isomeric galacturonic acid antiserum fails to protect against infection with Type II pneumococci, afford evidence that the hex-

uronic acid constituent of the Type II specific polysaccharide is probably glucuronic acid.

There remains still one perplexing fact to be considered. Although cellobiuronic, gentiobiuronic, and glucuronic acid antisera all confer passive immunity on mice against infection with Type II organisms, specific agglutinins and precipitins are not demonstrable in any of these antisera. Although the reactions of agglutination and precipitation are not demonstrable *in vitro* it is possible to show that the immune bodies combine with the organisms, for absorption of the sera[1] with encapsulated Type II pneumococci removes the protective antibodies, whereas absorption with the R or unencapsulated strain does not. On the basis of specific protective tests it is evident that it has been possible to produce in rabbits antibacterial sera with artificially compounded antigens containing saccharides of synthetic rather than of bacterial origin.

SUMMARY

1. Azoproteins containing structurally isomeric aldobionic acids evoke in rabbits antibodies which are both serologically and immunologically specific.

2. Antigens containing the azobenzyl glycosides of the synthetic gentiobiuronic and glucuronic acids evoke in rabbits antibodies which confer passive immunity on mice against infection with multiple lethal doses of virulent Type II pneumococci.

BIBLIOGRAPHY

1. Goebel, W. F., and Avery, O. T., *J. Exp. Med.*, 1931, **54**, 431, 437.
2. Goebel, W. F., *J. Exp. Med.*, 1939, **69**, 353.
3. Hotchkiss, R. D., and Goebel, W. F., *J. Biol. Chem.*, 1937, **121**, 195.
4. Hotchkiss, R. D., and Goebel, W. F., *J. Biol. Chem.*, 1936, **115**, 285.
5. (*a*) Helferich, B., and Klein, W., *Ann. Chem.*, 1926, **450**, 219. (*b*) Helferich, B., and Bredereck, H., *Ann. Chem.*, 1928, **465**, 166. (*c*) Helferich, B., and Rauch, H., *Ann. Chem.*, 1927, **455**, 168. (*d*) Helferich, B., and Sparmberg, G., *Ber. chem. Ges.*, 1933, **66**, 806.
6. Goebel, W. F., and Babers, F. H., *J. Biol. Chem.*, 1935, **111**, 347.
7. Reynolds, D. D., and Evans, W. L., *J. Am. Chem. Soc.*, 1938, **60**, 2559.
8. Isbell, H. S., *Bureau Standards J. Research*, 1930, **5**, 1179.
9. Vorhees, V., and Adams, R., *J. Am. Chem. Soc.*, 1922, **40**, 1397.
10. Brauns, D. H., *J. Am. Chem. Soc.*, 1927, **49**, 3170.
11. Goebel, W. F., and Hotchkiss, R. D., *J. Exp. Med.*, 1937, **66**, 191.
12. Brown, R., *J. Immunol.*, 1939, **37**, 445.
13. Goebel, W. F., *J. Biol. Chem.*, 1935, **110**, 391.
14. Landsteiner, K., The specificity of serological reactions, Springfield, Illinois, Charles C. Thomas, 1936.

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[1] This experiment has not been performed with cellobiuronic acid antiserum.

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CONSTITUENTS OF ELEMENTARY BODIES OF VACCINIA

I. CERTAIN BASIC ANALYSES AND OBSERVATIONS ON LIPID COMPONENTS OF THE VIRUS

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Five years ago preliminary chemical analyses of elementary bodies of vaccinia, consisting of an estimation of protein, fat, and ash, were reported from this laboratory (1). The subject was not pursued further at that time because the purity of the virus preparations was a matter of some doubt. Numerous developments within the past few years have eliminated much of the uncertainty on this score. For instance, more accurate methods for obtaining infective titers of virus have been developed (2) and applied to studies on vaccinia (3); moreover, the results of experiments from two different laboratories (4, 5) seem to indicate that a single infective unit of virus is capable of initiating infection under proper conditions; and, finally, a close correlation between the number of infective units and the estimated number of elementary bodies in properly purified preparations of vaccine virus has been observed (6). Therefore, we believe that the virus preparations are now known to be of sufficient purity to warrant a thorough investigation of their various constituents. The present report deals with certain basic chemical analyses on final preparations of virus and on various fractions discarded during the process of purification. In addition, the essential nature of several lipids in the structure of the elementary body will be considered.

Materials

Preparation of Elementary Bodies of Vaccinia.—Stock suspensions of elementary bodies of vaccinia were obtained by Craigie's technique (7) from rabbits infected cutaneously with the C.L. strain of virus. This strain of virus, which was employed by Craigie in the experiments reported in 1932, has been maintained in our laboratory since 1933. During this time it has been passaged on the average of once a month by the cutaneous inoculation in rabbits of washed elementary bodies. Stock suspensions of this washed virus were highly infectious when titered intracutaneously in rabbits. Each 0.25 cc. of the 40 cc. of suspension obtained from one rabbit contained from 10^9 to 10^{10} infective units when estimated by the 50 per cent end point method (2).

In order that there be no misunderstanding about the source of the several types of material analyzed in this work, a description of the technique of purification of the virus is essential. The fur from large, healthy chinchilla rabbits with unpigmented skins was removed, by means of an electric clipper equipped with No. 0000 blades, over an area extending from the neck to the rump and down both flanks. The skin, while being held taut, was gently scraped with the edge of a folded piece of 100 mesh bronze wire clasped in a hemostat. During the scraping of each area the skin and wire were moistened with a 1:5 dilution of a stock suspension of elementary bodies. About 5 cc. of the diluted virus suspension were applied to each rabbit, and when the procedure was finished the entire shaven area, except for a narrow margin of uninoculated skin, presented a uniform pink blush. Vigorous scraping which would result in the oozing of serum or frank bleeding was avoided. On the morning of the 3rd day—and it is important that the incubation period be limited to approximately 72 hours, because if it is extended the purification of the virus is less successful—the rabbit was sacrificed by intravenous injection of air, and the inoculated area of skin was quickly removed and stretched on a board. The surface was rinsed with ethyl ether and moistened with dilute phosphate buffer solution, pH 7.2. (Buffer solution, prepared according to McIlvaine's table, was diluted 1:50 with freshly boiled distilled water and autoclaved. Solutions, pH 7.2 after autoclaving, were prepared frequently and were discarded when the reaction became neutral or slightly acid.) The moistened skin was then covered with dilute buffer solution and gently scraped with a dull knife, a total of 25 cc. of the solution being used during the procedure. The material obtained was transferred to a pyrex tube, vigorously shaken, and then spun in a horizontal centrifuge at 3000 R.P.M. for 5 minutes. Floating fatty material and hair were removed with a swab and discarded. The opalescent supernatant fluid was poured off and saved, after which 12 cc. of dilute buffer solution were added to the sediment and the tube again shaken vigorously. Centrifugation at 3000 R.P.M. for 5 minutes was again employed and the supernatant fluid was added to that obtained in the previous step. The sediment, approximately 1.0 cc. of gray pulp, was ordinarily discarded, but in certain instances was saved for comparative chemical analyses. This will be referred to as "first horizontal sediment." The pooled supernatant fluids were again centrifuged at 3000 R.P.M. for 5 minutes. The 0.1 cc. of sediment from this run, which like the first horizontal sediment was usually discarded, was saved in some instances for chemical analyses. This material will be designated as "second horizontal sediment."

The pooled fluids resulting from horizontal centrifugation, rich in elementary bodies and relatively free of large particles of debris, were next spun at 3000 R.P.M. for 1 hour in flat tubes in the Swedish angle centrifuge kept in the cold room at 0°C. This procedure sedimented the virus. After the supernatant fluid had been poured off the sediment was resuspended in dilute buffer solution. In this manner the virus was sedimented three times. The material from each rabbit was finally resuspended in 40 cc. of dilute buffer solution and spun in a 50 cc. tube on an International centrifuge at 3000 R.P.M. for 40 minutes. Recently this step also has been carried out in the cold room. Supernatant fluid from this centrifugation will be referred to as "stock suspension" of elementary bodies. The sediment which resulted from this run and which was saved for study in several instances will be designated as "third" or "final horizontal sediment." Groups of 4 rabbits were used to prepare each lot of 160 cc. of elementary body suspension.

Lots of virus were titered within a few days after preparation by intracutaneous inoculation into rabbits of the Havana breed. During the past year, 2 areas were inoculated respectively with 0.25 cc. of the 10^{-7} and 10^{-8} dilutions of each lot of virus, while 8 areas were inoculated with the 10^{-9} and 10^{-10} dilutions, respectively. Various solutions have been employed for diluting the virus for titration, namely, dilute phosphate buffer solution, Locke's solution which contained 5 per cent fresh inactivated normal rabbit serum, a mixture of 60 per cent dilute phosphate buffer and 40 per cent Locke's solution to which was added 5 per cent rabbit serum, and, finally, a mixture of equal parts of 10 per cent dextrose solution and dilute phosphate buffer to which was added 5 per cent normal serum. The last 2 mixtures appeared to give the most consistent results.

Lots of virus suspension, with a few cc. of anhydrous ethyl ether added to inhibit growth of bacteria, were stored at 3°C. until 5 to 8 of them were collected; this usually required several weeks. The lots of elementary bodies were pooled, concentrated in the ultracentrifuge, and washed with dilute phosphate buffer solutions first at pH 6.0, and then at pH 8.0, and, finally, with several changes of distilled water, following which the virus was dried from the frozen state (6). After further dehydration over phosphorus pentoxide the preparations were weighed. The procedure for estimating the number of elementary bodies in a given pool from the dry weight of the material as well as the method of obtaining the infective unit-elementary body ratio of each preparation has been described in a recent paper (6) in which appeared data regarding the infectivity of 5 of the 11 pooled preparations analyzed in the work described in the present paper.

Methods of Chemical Assay

Because the microchemical methods employed in these analyses may serve in some instances to explain certain discrepancies between data to be presented here and those reported elsewhere (1, 8, 9), the procedures used, particularly those for fat fractionation studies, will be described in some detail.

Lipid Analyses.—Estimation of lipid by weighing alcohol-ether extractable material is subject to errors of great magnitude. For example, Folch and Van Slyke (10) and Christensen (11) have recently called attention to the occurrence of protein, amino acids, chlorides, phosphates, and urea in alcohol-ether extracts; these non-lipid materials were shown to account for as much as 80 per cent of the "fatty" material in some biological preparations. Methods of lipid analysis developed by Kirk, Page, and Van Slyke (12) and modified by Folch and Van Slyke (13) were employed throughout our work. In this method of fat fractionation due cognizance has been taken of non-lipid materials which contaminate alcohol-ether extracted lipids; furthermore, manometric methods afforded an analytical precision in the examination of the relatively small amounts of materials available which was not obtainable by other techniques. In our hands, these methods have shown that in some instances as much as 50 per cent of the alcohol-ether extractable substances of dried elementary bodies may be non-lipid in nature. Errors of this type were obviated by the use of non-polar solvents for re-extraction of the evaporated residue of the alcohol-ether extracts.

A representative lipid determination which serves to illustrate the methods employed is presented in the following protocol.

20 mg. of purified elementary body preparation, dried to constant weight, were extracted for 2 hours with 15 cc. of a boiling, 3:1 alcohol-ether mixture in a 25 cc. flask with a condenser of the Graham variety. Longer periods did not increase the yield of lipid, but increased considerably the amount of non-lipid substances. After cooling to room temperature, the mixture was filtered rapidly through a fat-free Seitz pad, and made up to 25 cc. in a volumetric flask with several successive portions of alcohol-ether mixture drawn through the filter pad. That no fat was lost in this manner was shown by the inability to recover lipid material from several discarded Seitz pads. 20 cc. were transferred to a beaker, and evaporated to dry residue on a water bath at 85°C. under partial vacuum. Reextraction of dry residue was carried out with several small portions of warm redistilled petrol ether which were then pooled and transferred to a sintered glass funnel and filtered into a 20 cc. volumetric flask. In this manner the original ratio of weight of elementary body preparation to volume of extraction fluid was maintained throughout successive fractionation. 2 cc. aliquots of the petrol ether extract were pipetted into combustion tubes, evaporated to dryness in the water bath, and combusted to carbon dioxide which was measured manometrically according to the method of Kirk, Page, and Van Slyke (12).

Total Lipid.—The total lipid values were calculated as mixed lipids.

Cholesterol.—1 cc. aliquots of the alcohol-ether extract were pipetted into combustion tubes and the cholesterol was precipitated as the digitonide. The precipitate was caught on a filter stick and combusted in the manner described for total lipid. The cholesterol was calculated from the carbon dioxide given by combustion of the cholesterol-digitonide complex.

Cholesterol Esters.—No increase in cholesterol was obtained after saponification, hence it was concluded that the cholesterol was present entirely as free, or non-esterified cholesterol.

Phospholipid.—Phospholipid was determined from the lipid phosphorus in the petrol ether extract. The phosphorus analyses were performed on a digest of the petrol ether residue, and the amount was determined by combustion of the strychnine phosphomolybdate precipitate, according to the method of Kirk, Page, and Van Slyke (12). The phosphorus-nitrogen ratio of the petrol ether residue was approximately 1:1, indicating that the phospholipid was lecithin.

Neutral Fat.—Neutral fat was estimated from the value for total lipid by subtracting the sum of the values for cholesterol and phospholipid.

Nitrogen.—Nitrogen was estimated by the Van Slyke procedure (14) following digestion with sulfuric acid and selenium oxychloride.

Amino Acids.—Alpha-amino acids were determined, following hydrolysis by 6 N hydrochloric acid, by the ninhydrin alpha-amino carboxyl technique of MacFadyen and Van Slyke (15). This method was particularly suitable for the small amounts of material available. 5 mg. of elementary bodies were sufficient for an accurate determination.

Cystine.—Cystine was estimated by the nitroprusside reaction (16). There was no inorganic sulfur, as shown by the benzidine precipitation method (17).

Reducing Sugars.—These were estimated by the method of Shaffer and Somogyi (18) following hydrolysis with 2 N hydrochloric acid.

Organic Phosphorus.—Organic phosphorus was determined by the method of Kirk

(19) on samples of dried virus prepared according to routine. Tests for inorganic phosphorus were negative on final preparations of elementary bodies. A correction was applied to the data obtained on horizontal sediments which contained appreciable amounts of inorganic phosphorus.

EXPERIMENTAL

Analyses of Final Preparations of Elementary Bodies

Dried material from eleven preparations of elementary bodies of vaccinia was subjected to chemical analysis. The purity of the different lots was of

TABLE I

Analyses of Elementary Bodies of Vaccinia

Lot	IU·EB ratio*	Phosphorus	Total nitrogen	Alpha-amino N after hydrolysis	Carbon	Lipid: Total lipid	Lipid: Cholesterol	Lipid: Phospholipid	Lipid: Neutral fat	Cystine after hydrolysis	Reducing sugars after hydrolysis†
		per cent	*per cent*	*per cent*	*per cent*	*per cent*	*per cent*	*per cent*	*per cent*	*per cent*	*per cent*
46	1:7.6	0.59	15.3	3.6	34.2	5.1	1.1	2.1	2.0		3.0
47	1:8.8	0.59	15.6	3.1	34.9	6.1	2.2	1.8	2.1	1.8	2.3
48	1:2.7	0.50	15.1	2.5	33.6	5.0	1.1	2.0	1.9	1.6	3.2
53	1:4.7	0.58	15.6	3.7	32.6	5.0	1.0	2.2	1.8		2.0
55	1:3.0	0.49	15.3	3.7	31.8	4.3	1.0	1.7	1.6	2.0	2.6
56	1:3.7	0.59	14.8	2.8	34.5	6.1	1.2	2.5	2.4		3.5
57	1:9.2	0.58	15.2	3.1	33.8	5.3	1.1	2.2	2.0		3.0
58	Not done	0.57	15.2	3.5	34.8	4.9	1.0	1.9	2.0		3.1
66	1:4.2	0.59	14.9	3.6	34.6	8.1	2.1	2.8	3.8		2.5
69	1:3.7	0.62	15.4	3.2	32.5	6.5	1.8	2.1	2.6	1.8	2.3
70	1:1.5	0.57	15.5	4.1	34.2	6.2	2.0	2.5	1.7	2.3	3.5
Average...	1:4.9	0.57	15.3	3.4	33.7	5.7	1.4	2.2	2.2	1.9	2.8

Values of all chemical analyses are expressed as per cent of dry weight.

* IU:EB ratio = infective unit-elementary body ratio.

† Expressed as glucose.

a relatively high order, since the infective unit-elementary body ratios varied between 1:1.5 and 1:9.2, averaging 1:4.9. Values for total nitrogen, alpha-amino nitrogen, carbon, carbohydrate, cystine, total lipid, cholesterol, phospholipid, and neutral fat were obtained and expressed as percentage of dry weight of the virus. The data are summarized in Table I.

The summarized data in Table I show a striking consistency. This is particularly evident in the analyses, *e.g.*, phosphorus, nitrogen, and carbon, which can be done directly and with considerable accuracy on the small amounts of material with which it was necessary to work. Virus lots 46 and 47, both of which contained 0.59 per cent phosphorus, had been previously analyzed for this element (9) by a different technique with resultant

values of 0.44 and 0.43 respectively. The phosphorus-nitrogen ratios of the phospholipid were approximately 1:1.

TABLE II

Successive Analyses on "Horizontal Sediments" Discarded during Preparation of Elementary Bodies

Sample from rabbit No.	Phosphorus	Total nitrogen	Alpha-amino N after hydrolysis	Carbon	Lipid				Cystine after hydrolysis	Reducing sugars after hydrolysis*
					Total lipid	Cholesterol	Phospholipid	Neutral fat		
	"First Horizontal Sediments"									
	per cent	*per cent*	*per cent*	*per cent*	*per cent*	*per cent*	*per cent*	*per cent*	*per cent*	*per cent*
1	1.01	11.1	2.1	35.1	11.2	2.1	1.6	7.5	4.5	1.1
2	1.06	12.8	2.2	36.8	12.1	1.9	1.2	9.0	3.9	2.2
3	1.00	12.2	2.7	36.9	11.6	2.2	1.8	7.2	4.2	1.1
4	1.01	11.7	1.7	34.2	9.8	1.6	2.0	6.2	3.9	2.3
Average......	1.02	12.0	2.2	35.8	11.2	2.0	1.7	7.5	4.1	1.7
	"Second Horizontal Sediments"									
1	0.91	13.7	1.9	34.5	8.2	1.8	1.2	5.2	1.6	1.1
2	0.81	13.8	2.0	34.8	7.6	1.6	1.2	5.2	1.2	2.1
3	0.72	12.1	2.4	36.2	9.7	1.8	1.5	6.4	1.0	1.1
4	0.61	12.2	1.4	36.2	7.9	1.5	1.8	4.6	1.6	1.2
Average......	0.76	13.0	1.9	35.4	8.4	1.7	1.4	5.4	1.4	1.4
	"Third (Final) Horizontal Sediments"									
1	0.61	12.8	1.9	32.6	7.1	1.8	1.2	4.2	1.2	1.2
2	0.59	13.5	1.8	31.8	7.0	1.2	1.2	4.6	1.5	2.1
3	0.70	13.0	1.7	34.2	8.0	1.9	2.0	4.1	1.2	1.4
4	0.68	13.9	1.9	33.1	6.9	1.2	1.7	4.0	1.6	2.3
Average......	0.65	13.3	1.8	32.9	7.2	1.5	1.5	4.2	1.4	1.8
	Final Preparation of Elementary Bodies Separated from Above Sediments									
	0.57	15.5	4.1	34.2	6.2	2.0	2.5	1.7	2.3	3.5

Values of all chemical analyses are expressed as per cent of dry weight.
* Expressed as glucose.

Chemical Nature of Material Discarded during Process of Purification of Elementary Bodies

In Table II are presented separate analytical data on three fractions of dermal pulp which were collected from 4 infected rabbits and which would ordinarily have been discarded during the process of purification of the virus; in addition, values on the final preparations of elementary bodies

obtained from these 4 animals are included. In order to avoid introducing inorganic phosphorus present in the ordinary buffer solution, the dermal pulp was suspended and washed with 0.01 molar lithium-veronal buffer solution, pH 7.9. It is at once apparent that the percentages of total and carboxyl nitrogen, and of reducing sugars progressively increased as the final or purified preparation of elementary bodies was approached. On the other hand, the percentages of organic phosphorus and of total and neutral fat progressively decreased in successive fractions that were separated, and were lowest in the final virus preparation. Cystine, which was present in large amounts in the "first horizontal sediment," was reduced in the "second and third horizontal sediments," but was again present in increased amounts in the final elementary body preparation.

In general, one can say that significant differences were noted in the amounts of phosphorus, total nitrogen, alpha-amino nitrogen, total lipid, neutral fat, reducing sugar, and cystine in the various types of materials discarded during the purification of the virus and in the amounts of these constituents in the final or purified preparation of elementary bodies. Furthermore, variations in the determinations on different lots of the same types of material stood out in contrast to the constancy observed in the values obtained in the final elementary body preparations.

The Rôle of Lipids in Elementary Bodies

The progressive diminution in the total lipids in the various fractions that were ordinarily discarded during the process of obtaining purified elementary bodies, strongly suggests that a value much in excess of 6.0 per cent for the final virus preparation indicates a contamination with non-essential fatty material. On the other hand, the failure to obtain a reduction in the lipid content of purified elementary bodies below 4.3 per cent is perhaps some evidence for assuming that this amount of fat may be an integral part of the virus. Two lines of investigation were followed to determine whether one or more of the lipid fractions obtained from elementary body preparations are integral constituents of the virus.

Extraction of Virus with Ethyl Ether.—Ethyl ether has long been known to have little or no inactivating effect on vaccine virus. In fact, stock suspensions of elementary bodies in buffer solution are saturated with ether to prevent bacterial growth during storage (7). Moreover, McFarlane and associates (8) have shown that extraction of dried virus with ethyl ether at room temperature does not reduce appreciably the infectivity of the material. We have also found no inactivation of the virus when it is extracted

with ethyl ether in the cold as evidenced by the results of the following experiment.

Elementary body pool No. 69, whose infective unit-elementary body ratio was 1:3.7, contained 407.0 mg. of dry material. 100 mg. were employed for chemical analyses, the results of which are presented in Table I. 301.7 mg., available for this experiment, had been stored under vacuum over phosphorus pentoxide at 0°C. during an interval of 6 weeks after having been dried from the frozen state. 12.5 mg. were removed for titrations of infectivity and stored under the same conditions; the remaining portion, 289.2 mg., was suspended in 50 cc. of cold anhydrous absolute ethyl ether and stored in a stoppered container at 0°C. for 48 hours; the suspension was thoroughly shaken on a number of occasions. The virus was sedimented from the ether by centrifugation in the angle centrifuge in the cold and again suspended in 50 cc. of ether. This extraction

TABLE III

Analyses of Elementary Bodies before and after Extraction with Ethyl Ether

	Elementary bodies before extraction	Elementary bodies after extraction	Ether extract residue
	per cent	*per cent*	*per cent*
Total lipid	6.5	5.0	1.62
Total nitrogen	15.4	15.6	0.00*
Cholesterol	1.8	0.00*	1.6
Phospholipid	2.1	2.2	0.00*
Neutral fat	2.6	2.8	±0.1

Values are expressed as per cent of dry weight of virus.
No appreciable loss of infectivity after extraction.
* Trace, insufficient quantity for estimation.

likewise proceeded for 48 hours at 0°C. when the suspension was again centrifuged. The supernatant ether was added to the first extract and saved. The ether extracted virus was dried under vacuum and weighed; 278.0 mg. were recovered. Hence, the material lost during extraction amounted to 11.2 mg. or 3.9 per cent of the original preparation. Results of analyses on the ethereal extract and on the treated virus are presented in Table III; for convenience the results obtained before extraction are repeated from Table I.

15 mg. of ether extracted virus were resuspended in 30 cc. of a dilute buffer solution containing 5 per cent of fresh normal inactivated rabbit serum, the final pH of the mixture being 7.7 (glass electrode). The method recommended by McFarlane and associates (8) was employed in resuspending the elementary bodies; this consisted of breaking up the aggregated clumps of elementary bodies in a TenBroeck grinder (20), an illustration of which was reproduced in their article. Instead of grinding entirely by hand, however, it was found expedient to insert the top of the pestle into a rubber stopper placed on the shaft of an ordinary stirring machine and to complete the process by mechanical power. In a similar manner, the 12.5 mg. of dry unextracted virus were suspended in 25 cc. of fluid. The method of resuspension was quite efficient, since the

majority of particles appeared to be monodispersed when smears stained by Morosow's technique (21) were examined. Moreover, each of the aggregates that remained was composed of only a few elementary bodies. Three rabbits were each inoculated intracutaneously with dilutions of both preparations and the results are summarized in Table IV.

The data summarized in Table IV show that no appreciable inactivation of virus occurred as the result of ether extraction. Both the dry virus and

TABLE IV

Titration of Resuspended Dry Elementary Bodies before and after Extraction with Ethyl Ether

Preparation	Log dilution	No. of inoculation	No. positive	No. negative	Accumulative positive	Accumulative negative	Per cent positive	Per cent negative	Infective units per ¼ cc. of suspension
							per cent	*per cent*	
Unextracted	−5	3	3	0	40	0	100	0	$10^{8.6}$, or 3.9 × 10^8
	−6	6	6	0	37	0	100	0	
	−7	10	10	0	31	0	100	0	
	−8	18	14	4	21	4	84	16	
	−9	18	6	12	7	16	30	70	
	−10	12	1	11	1	27	4	96	
Extracted	−5	3	3	0	38	0	100	0	$10^{8.5}$, or 3.2 × 10^8
	−6	6	6	0	35	0	100	0	
	−7	10	10	0	29	0	100	0	
	−8	18	12	6	19	6	76	24	
	−9	18	6	12	7	18	28	72	
	−10	12	1	11	1	29	3	97	

Infective unit-elementary body ratio of original material based on titration before drying was 1:3.7. Both resuspended preparations contained 0.5 mg. of dry material per cc. Therefore, on the basis of their infective titers, the IU:EB ratios were 1:241 and 1:294 for the unextracted and extracted portions, respectively.

the dry ether-extracted virus were resuspended in sufficient fluid to give a concentration of 0.5 mg. per cc. Therefore, since the infective units per 0.25 cc. were $10^{8.6}$ for the former and $10^{8.5}$ for the latter, their infective unit-elementary body ratios were 1:241 and 1:294, respectively. Sprunt and McDearman (5) have found that titration end points of vaccine virus suspensions obtained by this method are reproducible within 0.08 of a log dilution; the method is about as satisfactory in our hands. Although ether extraction did not affect the activity of the dry virus, it is to be noted that drying, storage, and resuspension result in an appreciable inactivation.

The analytical results presented in Table III indicate that at least one of the lipids, namely, cholesterol, is apparently not essential to the virus,

since it was removed almost completely without appreciably affecting the infective titer of the preparation. Saponification has not been found to increase the digitonide precipitating substance of the virus, hence it is likely that cholesterol is present only in the unesterified form. It seems reasonable to believe that cholesterol is merely adsorbed on the virus; this agrees with McFarlane's ideas (8).

Neutral fat and phospholipid were not appreciably altered by extraction with anhydrous ethyl ether in the cold, Table III. This would at least suggest that the neutral fat was not merely adsorbed on the virus particle; however, the essential nature of these lipids in the elementary body cannot be assessed from this experiment. Inasmuch as their removal from wet elementary bodies by extraction with alcohol and ether in the cold (22) or their removal from dry elementary body preparations by a mixture of these solvents (8) in each case results in inactivation of the virus, it might possibly be assumed that these constituents are essential to the integrity of the virus. Such a conclusion is not justified, however, because, as has already been pointed out (8), alcohol may inactivate the virus by some mechanism other than extraction of the lipid.

Action of Pancreatic Lipase on Vaccine Virus.—The effect of pancreatic lipase on the virus of vaccinia and on the lipids present in preparations of the virus was investigated in the following manner.

A water soluble globulin, possessing marked lipase activity when tested on tributyrin and triolein substrates, was prepared from a sodium chloride extract of acetone extracted pancreatin by repeated ammonium sulfate precipitation (23). 1 cc. of a water-clear solution of this material was sufficient to hydrolyze 0.05 gm. of tributyrin in 1 hour. 2 cc. of the solution were incubated for 1 hour with 50 mg. of vaccine virus, preparation No. 66, suspended in dilute phosphate buffer, 1 per cent sodium taurocholate being added to activate the lipase. At the end of the hour the virus was subjected to ultracentrifugation, and washed thoroughly in distilled water with repeated centrifugation. A portion was reserved for a study of infective titer, while the remainder, after being dried in the frozen state to constant weight, was subjected to chemical analysis. The results are summarized in Table V.

From the data in Table V it is at once apparent that pancreatic lipase, which had previously been shown to be active in the rapid hydrolysis of triolein and tributyrin, did not alter significantly the fat content of the virus preparation. The value for the percentage of neutral fat, determined before and after treatment on the basis of dry weight of virus, was, within the limits of the method, essentially unchanged. Moreover, the infectivity of the virus was not significantly altered by the action of lipase as shown by tests after treatment with active and heat-denatured enzyme; in one such

experiment the titer of both suspensions was $10^{-8.7}$. The latter finding is in accord with an observation made by Pirie (23) who found that the alleged inactivation of vaccine virus by lipase preparations was not due to a lipolytic action but to the presence of free fatty acids and lecithin in the material used as a source of lipase.

Two hypotheses immediately present themselves. In the first place, the fat might be bound in some manner whereby lipase is incapable of effecting hydrolysis. Such might be the case if the fat were built into a lipoprotein complex, or if it were stored within a membrane-like structure which

TABLE V

Action of Pancreatic Lipase on Elementary Bodies

Preparation	Total lipid	Cholesterol	Phospholipid	Neutral fat
	per cent	*per cent*	*per cent*	*per cent*
Before lipase treatment	8.1	2.1	2.8	3.8
After lipase treatment	8.0	1.9	2.6	3.5

Values are expressed as per cent of dry weight of virus.

TABLE VI

Hydrolysis by Pancreatic Lipase of Fat Extracted from Elementary Bodies

Amount of virus taken for experiment, *mg*	20.0
Amount of neutral fat extracted (3.8 per cent of virus), *mg*	0.76
Amount of fat recovered by wet extraction after lipase, *mg*	0.11
Amount of neutral fat hydrolyzed by lipase, *mg*	0.65
Neutral fat hydrolyzed in 4 hours, *per cent*	86.0

did not admit the lipase molecule. Secondly, the material represented as neutral fat might be peculiarly constructed and resistant to lipase hydrolysis. Such fats are known and have formed the basis of a recent study by Kelsey (24).

In order to test the second hypothesis, the following experiment was performed.

The petrol ether extract from 20 mg. of elementary bodies (preparation No. 66) was emulsified mechanically with 1 cc. of the lipase preparation described above and incubated at 38°C. Sodium taurocholate, sufficient for a final concentration of 0.01 per cent, was added for activation. A blank determination consisting of 1 cc. of lipase and the same amount of sodium taurocholate was set up and likewise incubated. After 4 hours the solutions were titrated with 0.005 N carbonate-free sodium alcoholate to a phenolphthalein end point. The material was next extracted in the wet with alcohol and ethyl ether, and the total unhydrolyzed lipid determined by combustion of the petrol ether soluble portion of the extract.

It is clear from the results summarized in Table VI that the neutral fat in vaccine virus is not unusual as regards its susceptibility to hydrolysis by pancreatic lipase. It would seem that in the intact elementary bodies the physical arrangement of the neutral fat is such as to render it incapable of enzymic hydrolysis.

DISCUSSION

The average values obtained in the chemical analyses of eleven lots of dry washed elementary bodies of vaccinia were the following: total nitrogen, 15.3 per cent; alpha-amino nitrogen after hydrolysis, 3.4 per cent; total carbon, 33.7 per cent; total phosphorus, 0.57 per cent; cholesterol, 1.4 per cent; phospholipid (lecithin), 2.2 per cent; neutral fat, 2.2 per cent; reducing sugars after hydrolysis, 2.8 per cent; cystine, 1.9 per cent.

A nitrogen value of 15.3 per cent is somewhat higher than heretofore reported for washed elementary bodies (1, 8, 25). The total amount of phosphorus is likewise slightly greater than that recently reported from this laboratory (9). The rise in our present figure for the amount of phosphorus is not, like the rise in the nitrogen value, dependent on increased purity of the virus preparations, but is to be explained on the basis of a more accurate microchemical technique. The present values for nitrogen and phosphorus in dried washed elementary bodies approach closely those obtained by McFarlane and associates (8) on their virus preparations after extraction with alcohol and ether. Our figures for the lipid content of dried washed elementary bodies are definitely lower than the ones recorded by the British workers. These discrepancies are due in part to methods used and in part to materials analyzed. As to the latter, it has been clearly demonstrated that the total lipid value of each successive "horizontal sediment" obtained during the process of purification falls progressively and is lowest in the final or purified virus preparations. Therefore, one is led to believe that total lipid and phospholipid values greater than 6.0 and 2.2 per cent, respectively, are indications of contamination by materials from crude pulp.

The present observations strongly suggest, but do not prove, that cholesterol is not an essential constituent of the elementary body, since it was readily removed by ether extraction without appreciably affecting the infectivity of the virus. On the other hand, neutral fat, which in the free state was soluble in ether and hydrolyzed by lipase, was not reduced in elementary bodies by treatment with these agents—agents that do not impair the infectivity of the virus. In addition, the amount of phospholipid in elementary bodies was not diminished by treatment with these agents. It remains to be shown whether neutral fat and phospholipid are

integral components of the virus particle; nevertheless, the evidence is consistent with the idea that they are important parts of its structure.

Phosphorus in the phospholipid constituent of elementary bodies represents only about 0.08 per cent of the total weight of the virus. Previous observations (8, 9) lead us to believe that the major portion of the non-lipid phosphorus, approximately 0.5 per cent of the virus, is to be found in the thymonucleic acid fraction of the active agent. Application of the usual conversion factor to this amount of non-lipid phosphorus shows that 5.0 per cent of the elementary body is thymonucleic acid. The content of carbohydrate in this amount of nucleic acid would be sufficient to account for most of the reducing sugars detected after hydrolysis of the elementary bodies. That part of the reducing sugar may be derived from sources other than desoxyribose is to be expected since small amounts of glucosamine have been found (8, 9) and since the heat stable soluble antigen of vaccinia, which can be extracted from dried virus (9), possibly contains some carbohydrate.

The relative consistency of the carbon values in the successive discarded materials and in the final virus preparations is not surprising. Amounts of cystine in the various fractions appear to show a trend different from those of the other constituents studied. As one would expect, this substance constitutes an appreciable amount, 4.1 per cent, of dermal pulp, since keratin contains as much as 18 per cent cystine; then the amount diminishes in the next two "horizontal sediments;" and finally it rises to about 2.0 per cent in the purified material.

SUMMARY

The results of chemical analyses reveal that it is possible to secure preparations of elementary bodies of vaccinia that possess a considerable uniformity in their chemical constituents. Furthermore, the amounts of certain of these constituents, namely, nitrogen, alpha-amino nitrogen after hydrolysis, phosphorus, total fat, phospholipid, neutral fat, reducing sugar after hydrolysis, and cystine, in the purified virus are significantly different from those in the various materials discarded during the process of purification.

The amounts of phospholipid and neutral fat in the virus preparations are not appreciably affected by extraction with ethyl ether or by digestion with lipase, procedures which do not inactivate the virus. Cholesterol, on the contrary, is apparently completely removed by these manipulations, and hence is not considered to play an important part in the economy of the virus.

BIBLIOGRAPHY

1. Hughes, T. P., Parker, R. F., and Rivers, T. M., *J. Exp. Med.*, 1935, **62,** 349.
2. Reed, L. J., and Muench, H., *Am. J. Hyg.*, 1938, **27,** 493.
3. Parker, R. F., and Rivers, T. M., *J. Exp. Med.*, 1936, **64,** 439.
4. Parker, R. F., *J. Exp. Med.*, 1938, **67,** 725.
5. Sprunt, D. H., and McDearman, S., *J. Immunol.*, 1940, **38,** 81.
6. Smadel, J. E., Rivers, T. M., and Pickels, E. G., *J. Exp. Med.*, 1939, **70,** 379.
7. (*a*) Craigie, J., *Brit. J. Exp. Path.*, 1932, **13,** 259. (*b*) Craigie, J., and Wishart, F. O., *Brit. J. Exp. Path.*, 1934, **15,** 390.
8. (*a*) McFarlane, A. S., and Macfarlane, M. G., *Nature*, 1939, **144,** 376. (*b*) McFarlane, A. S., Macfarlane, M. G., Amies, C. R., and Eagles, G. H., *Brit. J. Exp. Path.*, 1939, **20,** 485.
9. Smadel, J. E., Lavin, G. I., and Dubos, R. J., *J. Exp. Med.*, 1940, **71,** 373.
10. Folch, J., and Van Slyke, D. D., *J. Biol. Chem.*, 1939, **129,** 539.
11. Christensen, H. N., *J. Biol. Chem.*, 1939, **129,** 531.
12. Kirk, E., Page, I. H., and Van Slyke, D. D., *J. Biol. Chem.*, 1934, **106,** 203.
13. Folch, J., and Van Slyke, D. D., personal communication.
14. Van Slyke, D. D., and Kugel, V., *J. Biol. Chem.*, 1933, **102,** 489.
15. MacFadyen, D., and Van Slyke, D. D., personal communication. Method to be published.
16. Blankenstein, A., *Biochem. Z.*, 1930, **218,** 321.
17. Wakefield, E. G., *J. Biol. Chem.*, 1929, **81,** 713.
18. Shaffer, P. A., and Somogyi, M., *J. Biol. Chem.*, 1933, **100,** 695.
19. Kirk, E., *J. Biol. Chem.*, 1934, **106,** 191.
20. TenBroeck, C., *Science*, 1931, **74,** 98.
21. Morosow, M. A., *Centr. Bakt., 1. Abt., Orig.*, 1926, **100,** 385.
22. Smadel, J. E., and Rivers, T. M., unpublished data.
23. Pirie, A., *Brit. J. Exp. Path.*, 1935, **16,** 497.
24. Kelsey, F. E., *J. Biol. Chem.*, 1939, **130,** 195.
25. Macfarlane, M. G., and Salaman, M. H., *Brit. J. Exp. Path.*, 1938, **19,** 184.

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CONSTITUENTS OF ELEMENTARY BODIES OF VACCINIA

II. PROPERTIES OF NUCLEIC ACID OBTAINED FROM VACCINE VIRUS

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Highly purified preparations of elementary bodies of vaccinia contain constant amounts of fat, carbohydrate, and protein which occur in concentrations not materially different from those found in bacterial cells (1). In addition, evidence for the presence of nucleic acid in vaccine virus has been obtained through studies of ultraviolet absorption spectra of alkaline extracts of purified elementary bodies (2), and by means of the Feulgen reaction this acid has been identified as the thymus type. In order to establish more firmly the nature of the nucleic acid in vaccine virus and to extend the quantitative data obtained previously (2), the present study was carried out.

Material and Methods

The nucleic acid was obtained from elementary bodies which had already been extracted with ether and alcohol for studies of lipids (1). A complete description of the technique by which the purified elementary bodies were obtained has been recorded elsewhere (1, 3). A number of techniques for the isolation of nucleic acid from yeast and bacteria were compared before any attempt was made to isolate it from vaccine virus. The method of Levene (4), with certain minor modifications, proved most satisfactory for the study of the very small quantities of material at our disposal.

EXPERIMENTAL

Isolation of Nucleic Acid

After preliminary experiments nucleic acid from elementary bodies of vaccinia was isolated in the manner described in the following experiment.

548 mg. of dry, ether-alcohol extracted elementary bodies were treated with 50 cc. of 5 per cent sodium hydroxide. The mixture was heated for 30 minutes which resulted in the solution of the virus with the exception of a small residue. The hydrogen ion concentration was then adjusted to pH 7.5 with glacial acetic acid. 20 cc. of colloidal iron (5 per cent Fe_2O_3) were then added slowly with the immediate formation of a heavy precipitate. Filtration was carried out overnight at 4°C. The filtrate was reduced to 15 cc. *in vacuo* and treated with two volumes of absolute methyl alcohol containing 2 per

cent hydrochloric acid. A white precipitate formed immediately and after 10 minutes was collected by centrifugation and washed with methyl alcohol until a negative test for chloride was obtained. The precipitate was dried *in vacuo* over calcium chloride. 15.6 mg. of a brownish white powder were obtained.

Spectroscopic Characterization of the Nucleic Acid.—Certain spectroscopic studies of alkaline extracts of elementary bodies of vaccinia have been

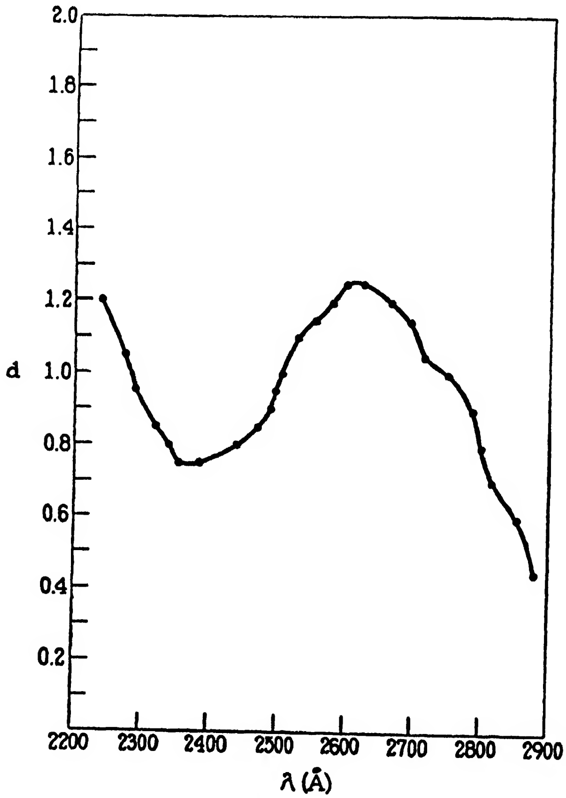

FIG. 1. Absorption curve of nucleic acid isolated from elementary bodies of vaccinia

previously reported (2). Caspersson (5), Lavin and Stanley (6), and others have recently extended the use of ultraviolet spectroscopy to the characterization of nucleic acid. The intense absorption of light by nucleic acids in the range from 2600 to 2650 Å has been shown to be due to purine and pyrimidine compounds, and is thus more or less unequivocal evidence of the presence or absence of nucleic acid if interfering substances are to some extent removed.

An absorption curve of nucleic acid isolated from vaccine virus, obtained

with the aid of a Spekker spectrophotometer and a small Hilger quartz spectrograph, is shown in Fig. 1. The concentration of nucleic acid from vaccine virus calculated from the phosphorus content was in close agreement with the concentration determined by spectrophotometric methods (2). Moreover, the absorption curve of nucleic acid isolated from the virus is similar to that obtained from a solution of nucleic acid isolated from thymus gland. It must be remembered that ultraviolet spectroscopy under the conditions employed does not distinguish between ribo- and thymonucleic acid.

Chemical Characterization of the Nucleic Acid.—Tests made on the material isolated in the manner described above showed it to be soluble and insoluble in dilute alkali and acid, respectively. It gave a negative biuret, negative ninhydrin, and a negative test for inorganic phosphate. A small amount boiled with 10 per cent sulfuric acid for 2 minutes gave a negative

TABLE I

Elementary Analysis of Nucleic Acid Isolated from Vaccine Virus

	Carbon	Nitrogen	Phosphorus
	per cent	*per cent*	*per cent*
Found in virus material	35.2	15.1	8.9
Calculated for a tetranucleotide	36.3	14.79	8.7

test for ribose with orcinol (Bial's test). Following hydrolysis a positive reaction was obtained by means of Schiff's base (Feulgen reaction) used in accordance with the method of Widström (7). In the absence of lipid and certain extraneous aldehydes, a positive reaction with this test is presumptive evidence of the presence of desoxyribose, a constituent of thymonucleic acid. A blue color obtained with diphenylamine (8) was similarly indicative of the presence of this pentose. A test for desoxyribose, in which the tryptophane reaction of Thomas was employed, was also positive (9).

A partial elementary analysis of a sample of the material, isolated from vaccine virus and dried to constant weight, revealed nitrogen, phosphorus, and carbon in very close agreement with analytical data presented by Levene for nucleic acid prepared from the thymus gland (4). The agreement of experimental values with the theoretical was quite satisfactory as indicated by the results recorded in Table I. Although too much importance cannot be attached to the results of elementary analysis of an amorphous substance, similarity of experimental and theoretical values together with the findings in preliminary qualitative tests described above.

were deemed indications of sufficient purity of the material under investigation to warrant further study.

A 5 mg. sample of the material was dissolved in a micro-reflux tube and heated on a boiling water bath with 10 per cent H_2SO_4 for 60 minutes. Concentrated ammonia was added gradually to a portion of the hot hydrolysate until the solution was quite alkaline. A fine white crystalline precipitate appeared almost immediately. This was separated by centrifugation and after being washed twice with hot ammonia water was dried *in vacuo*. Insufficient material was obtained for an elementary analysis, but qualitative tests for guanine were strongly positive. A larger sample of known thymonucleic acid, carried through the same procedure, gave similar crystals which analysis proved to be guanine.

The supernatant fluid from the guanine crystals obtained from vaccine virus was next boiled until free of ammonia. An excess of picric acid was then added and the mixture was allowed to stand; after several hours a few needle-like crystals separated from solution. There was insufficient quantity of crystals for elementary analysis, but qualitative tests for adenine picrate were positive.

Pyrimidine derivatives—cytosine, uracil, thymine—are separated from nucleic acid with great difficulty; consequently, the separation could not be accomplished on the minute quantity of material available. Nevertheless, certain color tests, introduced by Wheeler and Johnson (10*a*) and Harkins and Johnson (10*b*) are fairly satisfactory and were performed on the remaining portion of the hydrolysate obtained by the treatment of 5 mg. of nucleic acid with H_2SO_4. This portion was made neutral with NaOH after which bromine was added followed by an excess of barium hydroxide. A faint purple ring of barium dialurate was obtained. This color is given by cytosine and by uracil; hence, either one or both of these substances might have been present in the material examined.

The sample, on which the color test for uracil and cytosine was made, was next placed in a tube and distilled into a small quantity of water. Upon alkalinization, addition of *o*-aminobenzaldehyde, acidification with HCl, and realkalinization with sodium bicarbonate of the distillate, a faint blue fluorescence was obtained. This was due to 3-oxyquinaldine and indicated, according to Harkins and Johnson (10*b*), the presence of thymine in the material examined.

The experiment just described showed that guanine, adenine, and thymine were present in the nucleic acid examined. In addition, it was revealed that either one or both of the substances, cytosine and uracil, were also present. Inasmuch as thymine was present, it became obvious that the material under investigation contained thymonucleic acid; however, no quantitative estimation was possible. Uracil characterizes ribonucleic acid; but both uracil and cytosine react positively to the color tests used. Therefore, it was impossible to determine definitely from the results of the experiment whether yeast nucleic acid was present or not. Moreover, if it were present no idea of the amount was obtained. To clarify the situation and to get more information regarding the kind and amount of nucleic acid in our material, enzymatic studies were undertaken.

Enzymatic Characterization of the Nucleic Acid.—The use of enzymes in the differentiation of closely related substances is too well known to require emphasis. Jones (11), Dubos and Thompson (12), Schmidt and Levene (13), and Kunitz (14) have called attention to a heat stable enzyme in pancreas which depolymerizes ribonucleic acid. Nucleic acid containing desoxyribose, *i.e.*, thymonucleic acid, is apparently unaffected. Methods for the use of ribonuclease in the quantitative study of yeast nucleic acid have been described in detail by Schmidt and Levene (13). Through the kindness of Dr. Kunitz a sufficient supply of crystalline ribonuclease was made available for a study of its effect on the nucleic acid isolated from vaccine virus.

The method of Schmidt and Levene (13) for the determination of the activity of ribonuclease, useful as it is as a macro-analytical technique, was not found satisfactory for tests on the minute amounts of nucleic acid available. A technique developed by MacFadyen (15), however, in which unchanged nucleic acid is precipitated with uranyl chloride, was found to precipitate minute amounts of the unchanged acid from solution at pH 2. Nucleotides are precipitated only at higher pH values. By this technique it is possible to effect complete precipitation of less than 0.5 mg. of nucleic acid when the dilution is carefully controlled. Therefore, by means of MacFadyen's method and ribonuclease, a study of our material was made in the following manner.

2.5 mg. of nucleic acid isolated from vaccine virus were made up to 5 cc. with a buffer solution, pH 6.7, and incubated at 37°C. with 0.5 mg. of crystalline ribonuclease. Samples of 1 cc. were taken every 30 minutes for 2 hours, precipitated with 0.2 cc. of uranyl chloride-trichloracetic acid reagent, allowed to stand 10 minutes at room temperature, and then centrifuged at 2000 R.P.M. The supernatant fluid was decanted and the inside of the tube and precipitate were washed with 1 cc. of 10 per cent trichloracetic acid; centrifugation was again carried out at 2000 R.P.M., after which the supernatant fluid was discarded. The precipitate was digested and the phosphorus was determined according to the method of Kirk (16). The amounts of unchanged nucleic acid, *i.e.*, nucleic acid precipitable with the uranyl chloride-trichloracetic acid reagent, expressed in terms of milligrams of phosphorus have been plotted against time of enzymatic action as shown in Fig. 2. As a control on the activity of the enzyme, a known sample of yeast nucleic acid, purified according to the method of Levene, was used. The rapid depolymerization of the known yeast nucleic acid is indicated by the decreasing amounts of nucleic acid precipitable by uranyl chloride reagent which have also been plotted in Fig. 2.

The slight decrease in the amount of virus nucleic acid precipitable with the uranyl chloride-trichloracetic acid reagent after digestion with ribonuclease for 90 minutes (Fig. 2) may indicate the presence of a small amount of yeast nucleic acid. On the other hand, some spontaneous depolymeriza-

tion of the vaccine virus nucleic acid may have occurred independently of enzymatic activity. Known solutions of thymonucleic acid do not change appreciably, however, under similar conditions of incubation and precipitation. In any event, the results of the experiment recorded in Fig. 2 clearly indicate that at least 90 per cent of the material subjected to the action of ribonuclease was not yeast nucleic acid. It remained, however, to determine whether this 90 per cent was thymonucleic acid or not. This was accomplished by the use of diphenylamine reagent.

Quantitative Estimation of Nucleic Acid in Vaccine Virus

The carbohydrate released by the hydrolysis of thymonucleic acid gives an intense blue color with diphenylamine in acid solution under definite

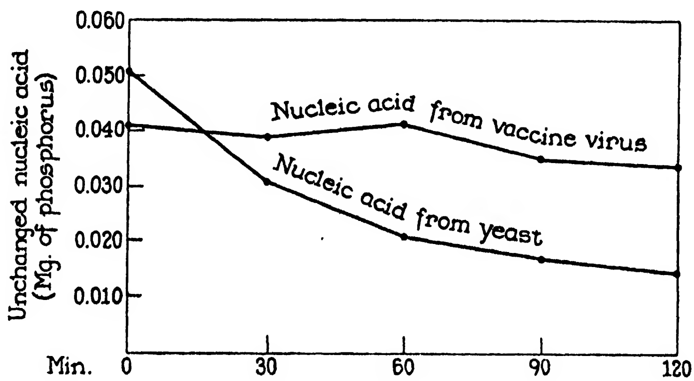

FIG. 2. Action of ribonuclease on nucleic acid isolated from vaccine virus and on nucleic acid isolated from yeast.

conditions (8). The color produced is stable and is proportional to the concentration of thymonucleic acid in the original solution. According to Bielschowsky and Klein (17) the color reaction is due to the thyminose content and is given only by the carbohydrates bound to the purine bases in the thymonucleic acid molecule (18). A modification of existing techniques, in which diphenylamine for the quantitative determination of thymonucleic acid is employed, resulted in a method suitable for the micro-determination of nucleic acid in vaccine virus as is indicated by the following experiment.

10 mg. of purified elementary bodies suspended in 2 cc. of 0.1 N HCl containing 1 per cent crystalline pepsin[1] were placed in a graduated centrifuge tube, shaken occasionally,

[1] The crystalline pepsin used in this study was generously supplied by Dr. J. H. Northrop. A study of the effects of crystalline enzymes on elementary bodies of vaccinia will be published soon.

and incubated at 37°C. for 2 hours. At the end of this time, a milky solution of the virus was obtained; no formed particles remained in suspension as demonstrated by Morosow's stain. 1 cc. of 5 N NaOH was next added; this resulted in complete clarification of the virus. The solution was next heated in a boiling water bath, the water lost by evaporation being replaced. The solution was then cooled, and 0.5 cc. of glacial acetic acid were added, followed by three volumes of methyl alcohol. A flocculent precipitate settled out on standing in the cold for 1 hour. The material was centrifuged for 5 minutes at 2000 R.P.M. and the supernatant fluid was decanted. To the precipitate were added 2 cc. of a reagent containing 1 per cent diphenylamine in 1:40 sulfuric acid-glacial acetic acid mixture and 1 cc. of water. The precipitate went into solution instantly, after which it was heated on a boiling water bath for 10 minutes, water being added to replace that lost on evaporation. A deep blue color appeared on cooling which was maximum after 30 minutes. This color was compared in a colorimeter with that given by a known concentration of thymonucleic acid treated in the same manner with

TABLE II

Quantitative Determination of Nucleic Acid in Elementary Bodies of Vaccinia

Lot No.	Amount of virus	Nucleic acid by color produced with diphenylamine	Phosphorus	Nucleic acid calculated from phosphorus content
	mg.	*per cent*	*per cent*	*per cent*
53	10.0	5.2	0.58	6.4
57	10.0	6.0	0.58	6.4
66	10.0	5.6	0.59	6.6
69	10.0	5.5	0.62	6.8
Average..........................		5.6	0.59	6.6

The values for phosphorus given in this table have been previously reported (1).

the diphenylamine reagent. In Table II are summarized the quantitative data obtained on specimens of virus assayed for thymonucleic acid in this manner. All determinations were done in duplicate.

From the data summarized in Table II it appears that 5.6 per cent of the elementary body of vaccinia consists of thymonucleic acid. If one assumes that the major part of the phosphorus from elementary bodies comes from the nucleic acid fraction, another set of calculations for nucleic acid is possible. Nucleic acid values calculated on this assumption are shown in Table II and agree favorably with direct estimation by means of the diphenylamine reagent.

DISCUSSION

Employing a relatively large amount of material, we have been able to obtain unequivocal evidence that the nucleic acid in vaccine virus is largely of the thymus variety. Failure of the major portion of isolated nucleic

acid to undergo depolymerization in the presence of crystalline ribonuclease demonstrates conclusively that not more than 10 per cent can be of the yeast type. The evidence for the presence of a small amount of ribonucleic acid in the vaccine virus rests solely thus far on the fact that there is a slight decrease in the nucleic acid precipitated with uranyl chloride-trichloracetic acid reagent following incubation with ribonuclease. In this study, as in previous ones, negative tests for ribose with Bial's reagent were obtained. *It is true that ribonucleic acid is more soluble than thymonucleic acid, a fact which may have resulted in proportionally greater losses of the ribonucleic acid in the process of isolation of our material.*

Although on previous occasions it has been stated that thymonucleic acid occurs in the viruses of vaccinia and psittacosis (2, 19), the data presented at this time, so far as we are aware, represent the most conclusive evidence that this type of nucleic acid occurs in a virus. This information is particularly interesting in view of the extensive work on nucleic acid in other viruses (6, 20–23) in which the ribonucleic type was found. The biological significance of our findings is not at the moment obvious.

SUMMARY

It has been possible by means of classical chemical methods to isolate and to characterize to some extent the nucleic acid of elementary bodies of vaccinia.

Determination by means of diphenylamine reagent revealed that the major part of the nucleic acid was of the thymus type. This was further substantiated by its stability in the presence of ribonuclease, less than 10 per cent undergoing depolymerization during prolonged incubation at 37°C.

By the technique employed, at least 5.6 per cent of the virus was shown to be thymonucleic acid. This amount agreed favorably with the value calculated from the non-lipid organic phosphorus of elementary bodies on the assumption that the phosphorus bound in the organic form was derived principally from nucleic acid.

BIBLIOGRAPHY

1. Hoagland, C. L., Smadel, J. E., and Rivers, T. M., *J. Exp. Med.*, 1940, **71**, 737.
2. Smadel, J. E., Lavin, G. I., and Dubos, R. J., *J. Exp. Med.*, 1940, **71**, 373.
3. Smadel, J. E., Rivers, T. M., and Pickels, E. G., *J. Exp. Med.*, 1939, **70**, 379.
4. Levene, P. A., *J. Biol. Chem.*, 1922, **53**, 441.
5. Caspersson, T., *Skand. Arch. Physiol.*, 1936, **73**, suppl. 8.
6. Lavin, G. I., and Stanley, W. M., *J. Biol. Chem.*, 1937, **118**, 269.
7. Widström, G., *Biochem. Z.*, 1928, **199**, 298.

8. Dische, Z., *Mikrochemie*, 1930, **8**, 4.
9. Thomas, P., *Z. physiol. Chem.*, 1931, **199**, 10.
10. (*a*) Wheeler, N. L., and Johnson, T. B., *J. Biol. Chem.*, 1907, **3**, 183.
(*b*) Harkins, H. H., and Johnson, T. B., *J. Am. Chem. Soc.*, 1929, **51**, 1240.
11. Jones, W., *Am. J. Physiol.*, 1920, **52**, 203.
12. Dubos, R. J., and Thompson, R. H. S., *J. Biol. Chem.*, 1938, **124**, 501.
13. Schmidt, G., and Levene, P. A., *J. Biol. Chem.*, 1938, **126**, 423.
14. Kunitz, M., *Science*, 1939, **90**, 112.
15. MacFadyen, D. A., *J. Biol. Chem.*, 1934, **107**, 297.
16. Kirk, E., *J. Biol. Chem.*, 1934, **106**, 191.
17. Bielschowsky, F., and Klein, W., *Z. physiol. Chem.*, 1932, **207**, 202.
18. Snell, F. D., and Snell, C. T., Colorimetric methods of analysis, New York, D. Van Nostrand Co., Inc., 1937, **2**, 274.
19. Robinow, C. F., and Bland, J. O. W., *Nature*, 1938, **142**, 721.
20. Bawden, F. C., and Pirie, N. W., *Proc. Roy. Soc. London, Series B*, 1937, **123**, 274.
21. Lavin, G. I., Loring, H. S., and Stanley, W. M., *J. Biol. Chem.*, 1939, **130**, 259.
22. Claude, A., *Science*, 1938, **87**, 467.
23. Bawden, F. C., Plant viruses and virus diseases, *Chronica Botanica*, Leiden, 1939, **5**, Chapter 9.

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INFECTIOUS MYXOMATOSIS OF RABBITS

II. Demonstration of a Second Soluble Antigen Associated with the Disease

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(Received for publication, May 24, 1940)

An earlier communication which dealt with a soluble antigen of infectious myxomatosis (1), contained data indicating the presence of a second serologically active substance in the lesions and in the serum of animals acutely ill with this disease. The observations reported at this time augment the earlier findings and indicate that two serologically distinct soluble antigens are associated with infectious myxomatosis.

Materials and Methods

Source of Soluble Antigens.—Two kinds of extracts of serologically active soluble substances have been used. One, filtered extract of infected skin, is prepared from the skin lesions produced in rabbits by the intradermal inoculation of infective material. The lesions are removed when fully developed but not necrotic, usually on the 6th day after inoculation, macerated in a meat grinder and stored at 0°C. in physiological salt solution. Ether is added to prevent bacterial growth. After periods varying from several days to several months, the fluid is cleared of large particles and passed through a Seitz filter which effectively removes the virus. The second source of soluble antigens is blood drawn by cardiac puncture from rabbits on the 5th or 6th day after the inception of an extensive infection of the skin; the serum is separated and filtered through a Seitz pad.

Preparation of Partially Purified Soluble Antigen (Fraction A).—In a previous report, the partial purification of a soluble antigen associated with myxoma has been described (1). The procedure involves precipitation of virus-free filtrates of myxomatous material in 50 per cent saturated solutions of ammonium sulfate. The precipitate, which contains the active principle, is dissolved in water and freed of ammonium sulfate by dialysis overnight against running water. The solution is then brought to pH 4.5 and ammonium sulfate is added to a concentration of 30 per cent. The precipitate which is obtained is dissolved and dialyzed against water. This solution constitutes fraction A.

Immune Sera.—Fibromyxoma serum was collected from rabbits which had been repeatedly inoculated with myxoma virus after recovery from an infection with fibroma virus. Fibroma serum was obtained from rabbits a month after inoculation with fibroma virus. Myxoma serum was collected from rabbits which had survived an attack of myxomatosis; two samples were available, one of which was supplied by Dr. R. F. Parker. Anti-soluble substance serum 9212, a pooled serum, was obtained

from rabbits following the injection of a partially purified extract (fraction A) of skin. These various sera were used in work previously reported (1). Sera from normal rabbits or from rabbits recovered from vaccinal infections were used to control the specificity of reactions.

Precipitin Reactions.—As a rule, graded dilutions of the antigen to be tested were mixed with constant amounts of immune sera; occasionally, however, graded dilutions of antisera were added to constant amounts of antigen. All test mixtures were incubated in closed racks overnight at 50°C.

EXPERIMENTAL

The previous experiments (1) that had suggested the presence of a second specific soluble substance in infectious myxomatosis were made with pooled antiserum which had been prepared in rabbits by the injection of partially purified virus-free material (fraction A) from myxomatous skin lesions. This antiserum, 9212, after absorption with fraction A no longer reacted with this antigen, but still precipitated with serum from rabbits moribund with infectious myxomatosis. For the sake of simplicity, the soluble antigen which has already been described in considerable detail (1) will henceforth be referred to as antigen A; the soluble substance which reacted with the antiserum absorbed free of A antibodies will be designated antigen B.

Two observations on antigen B were recorded in the studies on the characteristics of the first soluble antigen (1), and, since both have been confirmed, they may be briefly summarized. Antigen B, like antigen A, is heat labile for, after being heated at 56°C. for ½ hour, it no longer precipitates with its specific antibody. Furthermore, antigen B is discarded or inactivated during the procedure employed for the purification of antigen A.

Separation of the Two Soluble Antigens

It seemed logical to determine at what stage antigen B was lost in process of purifying antigen A. Moreover, it was obviously desirable to obtain solutions of each antigen uncontaminated by the other. Accordingly, crude materials containing the soluble substances were subjected to the method of fractionation previously employed. The presence or absence of the two soluble antigens in material obtained at each step in the fractionation was established by two sets of precipitin titrations. One test was made with antiserum 9212, which contained both A and B antibodies, and the other with antiserum 9212 which had been absorbed free of A antibodies. A representative experiment is detailed below.

42 cc. of filtered serum obtained from a rabbit moribund with myxoma were mixed with 42 cc. of a saturated solution of ammonium sulfate. The white precipitate which

formed was removed by centrifugation, dissolved in dilute phosphate buffer and dialyzed overnight against running water. The supernatant solution, containing material not precipitated in a concentration of 50 per cent ammonium sulfate, was dialyzed in a similar manner and each dialysate, after addition of NaCl to physiological concentration, was tested for precipitinogens with antiserum 9212 which contained A and B antibodies, and with another portion of this antiserum which had been absorbed free of A antibodies. The globulin fraction contained both precipitable substances; the solution of albumin contained only a negligible amount of serologically active material. Further fractionation of the globulin was carried out as follows: the dialysate was brought to pH 4.5 by the addition of N HCl, and a sufficient amount of a saturated solution of ammonium sulfate was added to bring the final concentration to 30 per cent saturation. The precipitate which formed was removed by centrifugation, dissolved in dilute buffer and dialyzed against running water overnight. The supernatant fluid was neutralized and enough of a saturated solution of ammonium sulfate was added to make a final concentration of 50 per cent saturation. The precipitate which formed was removed by centrifugation, dissolved in dilute buffer and dialyzed. The material precipitated at 30 per cent saturation with ammonium sulfate and that precipitated between 30 and 50 per cent saturation were about equal in amount and similar in appearance. Precipitin tests were performed with solutions of these fractions after the NaCl concentration had been brought to 0.85 per cent.

The data provided in the protocol and summarized in Table I indicate that both antigens were present in the fraction of the serum filtrate that was insoluble in a 50 per cent saturated solution of ammonium sulfate. Further fractionation of the globulin by precipitation in a 30 per cent saturated solution of ammonium sulfate at pH 4.5 brought about a separation of the two antigens: antigen A was insoluble in this concentration of the electrolyte while antigen B remained in solution and was subsequently precipitated by raising the concentration of ammonium sulfate to 50 per cent saturation. Similar results were obtained when the same type of procedure was applied to a filtrate of crude extract of myxomatous skin which was known to contain both antigens.

Immunization of Rabbits with Partially Purified and Whole Extracts of Myxomatous Skin and with Whole Serum from Acutely Ill Rabbits

According to the results of precipitin tests, a single fractionation with ammonium sulfate generally resulted in a sharp separation of antigens A and B, yet the employment of this method apparently left the preparation of antigen A contaminated with immunologically significant amounts of antigen B. It has already been pointed out that antiserum 9212, obtained by injecting rabbits with fraction A of an extract of myxomatous skin, contained antibodies against both types of soluble antigen. Hence, the injected material may be assumed to have contained antigen B although this substance was unrecognized at the time and therefore not sought for.

At the beginning of the present work, before the identity of antigen B appeared well established, it seemed desirable to determine whether antisera similar to 9212 could be regularly obtained. Accordingly, rabbits were injected with preparations apparently free of antigen B and with preparations containing both antigens A and B. Four rabbits were immunized in a manner similar to that employed in a previous experiment (1), that is, by means of a series of injections of partially purified extract

TABLE I

Precipitin Reactions of Fractions of Serum Antigen

Fraction of serum antigen	Immune serum 1:8	Dilution of antigen						
		Undiluted	1:2	1:4	1:8	1:16	1:32	1:64
Total albumin	9212 (anti A and B)	–	–	–	–	–	–	–
	9212 (absorbed with A)	–	–	–	–	–	–	–
Total globulin	9212 (anti A and B)		++++	++++	++++	+++	±	–
	9212 (absorbed with A)		++++	++++	++++	+	–	–
Globulin insoluble in 30 per cent ammonium sulfate at pH 4.5 (fraction A)	Normal	–	–	–	–	–	–	–
	Fibromyxoma	+	±	–	++++	+++	++	±
	9212 (anti A and B)	+++	++++	++++	++++	+++	+	–
	9212 (absorbed with A)	++	–	–	–	–	–	–
Globulin insoluble in 50 per cent ammonium sulfate at pH 7.0 (fraction B)	Normal	–	–	–	–	–	–	–
	Fibromyxoma	++	–	–	–	–	–	–
	9212 (anti A and B)	++++	++++	++++	++++	–	–	–
	9212 (absorbed with A)	+++	++++	++++	–	–	–	–

+'s indicate degree of precipitation.

(fraction A) of myxomatous skin. This immunizing material was obtained in the manner described and was shown to possess a high titer when tested for precipitinogens with antiserum 9212 (anti-soluble substance serum containing A and B antibodies); moreover, the material injected into the rabbits was shown to absorb from antiserum 9212 all antibodies against A and to leave those against B. Two additional groups of 4 rabbits each were immunized; one group received whole filtered extract of myxomatous skin, while the other was given whole filtered serum from rabbits moribund with myxomatosis. Both these immunizing materials were known to contain antigens A and B. The rabbits were bled at intervals and their sera tested

TABLE II

Precipitin Reactions with Antigens A and B and Sera from Rabbits Immunized with Three Preparations of Soluble Antigen

Immune serum	Material used for immunization	Test antigen	Dilution of antiserum						Test antigen	Dilution of antiserum					
			Undiluted	1:2	1:4	1:8	1:16	1:32		Undiluted	1:2	1:4	1:8	1:16	1·32
9212	Fraction A of skin	Fraction A of skin 1:6	++++	++++	++++	++++	+++	–	Fraction B of skin 1:8	++++	++++	++++	++++	+++	–
68	Whole filtrate of serum (containing antigens A and B)		++++	++++	++++	++++	++++	++++		+++	+	–	–	–	–
69			++++	++++	++++	++++	++++	++++		++++	+++	+++	–	–	–
70			++++	++++	++++	++++	++++	++++		+++	–	–	–	–	–
71			++++	++++	++++	++++	++++	++		±	+	++	–	–	–
74	Whole filtered extract of skin (containing antigens A and B)		++++	++++	++++	++++	++++	+		±	–	–	–	–	–
75			++++	++++	++++	++++	+++	±		++++	++++	++++	++++	+	–
76	Fraction A of skin		++	+++	++++	++	–	–		+++	++++	++	–	–	–
77			±	+++	+++	+++	++	–		++++	++++	++++	±	–	–
78			++++	++++	++++	++++	+++	++		++++	++++	++++	+++	–	–
79			++++	++++	++++	+++	++++	+++		++++	++++	++	±	–	–

+'s indicate degree of precipitation.

for the presence of precipitating antibodies against antigens A and B. The titers obtained at the end of the period of injection are recorded in Table II.

Examination of Table II reveals that the 4 rabbits (68–71) injected with whole filtered serum produced antibodies in high titer against antigen A but in much lower concentration for antigen B. Furthermore, of the 4 rabbits receiving extract of whole filtered skin only 2 (74, 75) developed antibodies against either of the antigens in concentration high enough to be detected and only 1 (75) possessed antibodies against B. *On the other hand, the injection of fraction A, which contained no demonstrable antigen B, induced the development of both A and B antibodies in the rabbits receiving it (76–79).* This finding confirms our previous experience in the production of antiserum 9212. Moreover, sera 76–79, when absorbed with partially purified extract of skin (fraction A), behaved in a manner similar to antiserum 9212, *i.e.*, they still precipitated with whole serum which contained both antigens A and B and with fraction B of skin and serum.

Inhibition of Antibodies by Non-Precipitable Forms of Antigens A and B

The relative instability of partially purified antigen A, even when dried from the frozen state and stored in the cold, has been noted (1) in preparations tested for precipitinogens. It seemed possible that slightly changed forms of the soluble antigen might still combine with their specific antibodies although visible precipitation did not occur. The ability of non-precipitable forms of the antigens to inhibit their antibodies was investigated in the manner outlined below.

An active fraction A of skin antigen which had been heated at 56°C. for 1 hour and which no longer precipitated with antiserum 9212 was added to an equal volume of this serum which contained antibodies against A and B. The mixture was incubated at 56°C. for 1 hour and stored overnight at 5°C. No precipitation occurred and the treated antiserum was tested for its activity with antigens A and B. In a similar manner an active fraction B of skin antigen, heated at 56°C. for 1 hour, was found to give no precipitate when mixed with antiserum 78 known to contain A and B antibodies. Equal parts of the heated antigen and the antiserum 78 were mixed, incubated at 56°C. for 1 hour, stored at 5°C. overnight and then tested for precipitating antibodies with antigens A and B. The results of the titrations are presented in Table III.

It is apparent from the results presented in Table III that the solutions of the two antigens which had completely lost their power to precipitate with antibodies as a result of heating at 56°C. were capable of specifically inhibiting their respective antibodies. Similar tests carried out with materials from rabbits infected with vaccine virus did not result in inhibition of antibodies against either antigen A or B of myxomatosis. Furthermore,

heated preparations of myxoma antigens did not inhibit the precipitin reactions of vaccinia. Specific inhibition of antivaccinal antibodies has been demonstrated, however, with degraded soluble antigens of vaccinia (2).

Occurrence of Antigen B in Infected Animals

The observations recorded indicate that two soluble antigens can be identified in myxomatous tissue of rabbits and in the sera of animals during the height of the disease. There seemed little reason to believe that the

TABLE III

Precipitin Reactions of Anti-Soluble Substance Sera after Treatment with Non-Precipitable Antigen

Immune serum	Test antigen	Dilution of antigen					
		1:2	1:4	1:8	1:16	1:32	1:64
9212 untreated (anti A and B)	Fraction A	++	+++	++	++	++	−
	Fraction B	++++	++++	++++	++	−	−
9212 treated with non-precipitable A	Fraction A	−	−	−	−	−	−
	Fraction B	++++	+++	++	+	−	−
		Dilution of antiserum					
		1:2	1:4	1:8	1:16	1:32	1:64
78 untreated (anti A and B)	Fraction A	++++	++++	++++	+++	+	−
	Fraction B	++++	++++	++++	−	−	−
78 treated with non-precipitable B	Fraction A		++++	++++	++++	−	−
	Fraction B		−	−	−	−	−

+'s indicate degree of precipitation.

second antigenic substance was formed during the process of manipulation incident to purification of these substances; nevertheless, this possibility had to be considered. Two types of evidence were obtained which demonstrated the occurrence of antigen B in the natural infection.

The first kind of evidence consisted of the detection of antigen B in the serum of rabbits acutely ill with myxomatosis. In earlier work, at a time when antigen B had not been recognized, it appeared that the first demonstrated specific soluble substance associated with myxoma (antigen A) was sometimes absent from samples of sera taken from moribund animals or was present in low concentration. Such sera, which contained no demon-

strable antigen A, were often found to contain large amounts of precipitable substance (antigen B) when tested with antisera known to precipitate with

TABLE IV

Titer of Precipitinogens (A and B) in Whole Filtrates of Myxomatous Serum Tested with Fibromyxoma Serum and Anti-Soluble Substance Serum 78

Fresh filtrate of serum	Immune serum 1:8	Dilution of antigen							Remarks
		1:2	1:4	1:8	1:16	1:32	1:64	1:128	
813	Fibromyxoma	+	±	+++	++++	++++	+++	±	Antigen A present
	78	+	++++	++++	++++	++++	++++	++	
649	Fibromyxoma	–	–	–	–	–	–	–	Only antigen B present
	78	++++	++++	++++	+++	++	–	–	
1	Fibromyxoma	–	–	–	–	–	–	–	Only antigen B present
	78	+++	++++	++++	++++	–	–	–	
653	Fibromyxoma	–	–	–	–	–	–	–	Only antigen B present
	78	++	++++	+++	++	+	–	–	

+'s indicate degree of precipitation.

Fibromyxoma serum gives precipitates only with antigen A, while anti-soluble substance serum 78 reacts with antigens A and B.

TABLE V

Precipitin Reactions of Two Samples of Myxoma Serum and Two Samples of Fibroma Serum with Antigen A and Antigen B

Antigen	Immune serum	Dilution of antigen					
		Undiluted	1:2	1:4	1:8	1:16	1:32
Fraction A	Myxoma 1	++++	++++	++++	++++	++	–
	Myxoma 2	++++	++++	++++	++++	++++	+
	Fibroma 1	++++	++++	++++	+	–	–
	Fibroma 2	++++	++++	++++	++	–	–
Fraction B	Myxoma 1	++++	+++	+	±	–	–
	Myxoma 2	++	–	–	–	–	–
	Fibroma 1	++++	+++	+	–	–	–
	Fibroma 2	±	–	–	–	–	–

+'s indicate degree of precipitation.

both A and B. These sera received no treatment except filtration through Seitz pads, hence the formation of antigen B by denaturation of a native substance in the serum seemed unlikely. The results of precipitin titrations obtained with sera containing both antigens A and B, and with others containing only antigen B are illustrated in Table IV.

The second type of evidence was furnished by the detection of B antibodies in the sera of two animals following recovery from infectious myxomatosis. It is of interest to note that B antibodies were also demonstrated in the serum of one of two rabbits convalescent from fibromatosis. The results of titrations with sera from these 4 animals are presented in Table V. The occurrence of B antibodies in convalescent animals may be taken as evidence that antigen B is present during the acute infection.

DISCUSSION

Infected tissue and serum from rabbits moribund with myxomatosis apparently contain two specific soluble antigens which have been designated A and B. Both antigens are inactivated at 56°C. and both are recoverable with the globulin fraction of solutions containing them. Antigen A is insoluble in a 30 per cent saturated solution of ammonium sulfate at pH 4.5, while antigen B is soluble in this concentration but precipitates in a half saturated solution of the salt. Both antigens readily lose their ability to flocculate in the presence of their specific antibodies. Nevertheless, non-precipitable forms of both antigens are capable of inhibiting their specific antibodies. The sera of certain rabbits convalescent from infection with fibromatosis and myxomatosis contain antibodies against both antigens.

The fibromyxoma serum used throughout an earlier series of experiments (1) contained adequate amounts of A antibodies, but, in the dilutions employed, failed to react appreciably with antigen B. This latter fact undoubtedly facilitated the studies dealing with the characteristics of antigen A and with the development of methods for its partial purification. On the other hand, this lack of B antibodies in the fibromyxoma serum delayed the identification of antigen B.

Antigens A and B are immunologically distinct and apparently can be separated by simple physical means; nevertheless, it has been observed that rabbits injected with preparations of antigen A regularly develop both A and B antibodies. Indeed, such a procedure more uniformly induces the production of B antibodies than does immunization with crude materials rich in both serologically active substances. An explanation of these phenomena is not at hand. One point is obvious, however, that is the lack of purity of preparations of antigen A. These undoubtedly contain antigen B in amounts which may be comparatively small but are still immunologically significant. If antigen B in small amounts is capable of eliciting antibody formation, then why does immunization with crude preparations of skin or serum containing large amounts of this substance fail to call

forth B antibodies? Only conjectures can be offered; one of the more obvious is that some one or more of the extraneous substances in the crude preparations interferes with the ability of antigen B to stimulate antibody production.

SUMMARY

A second soluble antigen, separable from the virus, occurs in extracts of infected skin and in the serum of rabbits acutely ill with infectious myxomatosis. Like the first antigen (A), the second (B) is heat labile and has certain characteristics of a globulin. The two antigens precipitate in different concentrations of ammonium sulfate and can be separated by this method. Neither of the antigens after being heated at 56°C. precipitates in the presence of specific antibody but each is capable of inhibiting the activity of its antibody.

BIBLIOGRAPHY

1. Rivers, T. M., Ward, S. M., and Smadel, J. E., *J. Exp. Med.*, 1939, **69**, 31.
2. Smadel, J. E., and Rivers, T. M., *J. Bact.*, 1940, **39**, 39.

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PANTOTHENIC ACID DIPHOSPHATE

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Since the isolation of pantothenic acid in pure form,[1,2] and the publication of its structure and synthesis,[2] the problem of its rôle in biochemical reactions has received attention. Many of the water-soluble vitamins take part in enzymic reactions as phosphate esters, and the possibility that pantothenic acid phosphates might be of significance was considered. Pantothenic acid acetate[3] and some other derivatives in which the hydroxyl groups are covered are inactive biologically. If the phosphates should be active, one might conclude that either the test organism contained a phosphatase capable of splitting the ester or that the ester itself took part in cell metabolism.

Since details of the synthesis of pantothenic acid have not yet appeared, our method of obtaining the *dl* acid will be indicated. β,β-Dimethyl-α,γ-dihydroxybutyric acid was made according to Kuhn and Neustadter[4] and its barium salt was acetylated and converted to the acid chloride with thionyl chloride. Union with β-alanine ethyl ester and selective hydrolysis of the ester linkages were performed as previously described.[5] The resulting pantothenic acid was converted to the barium salt with $BaCO_3$ and obtained as a white powder by adding ether to its alcoholic solution. Calculated for $C_{18}H_{32}O_{10}N_2Ba$, Ba 23.9; found, 23.9. 650 mg. of pantothenic acid in 10 cc. of ice-cold pyridine were treated with 1 gm. of $POCl_3$. After 1 hour, the mixture was concentrated to dryness under reduced pressure, and taken up in cold water. The solution was diluted and treated with 20 gm. of

[1] Woolley, D. W., *Science*, **91**, 245 (1940).

[2] Williams, R. J., and Major, R. T., *Science*, **91**, 246 (1940).

[3] Woolley, D. W., Waisman, H. A., Mickelsen, O., and Elvehjem, C. A., *J. Biol. Chem.*, **125**, 715 (1938).

[4] Kuhn, M., and Neustadter, V., *Monatsh. Chem.*, **39**, 293 (1918).

[5] Woolley, D. W., Waisman, H. A., and Elvehjem, C. A., *J. Biol. Chem.*, **129**, 673 (1939).

norit A. The norit was washed with alcohol and an aqueous solution of the eluate was neutralized with $Ba(OH)_2$. The barium salt was obtained as a fine, white powder by adding alcohol to its aqueous solution. Yield, 700 mg. Calculated for $C_{18}H_{28}O_{22}N_2P_4Ba_5$, P 8.6, Ba 47.8; found, P 8.5, Ba 47.7. Attempts to prepare a crystalline brucine salt were unsuccessful.

Substance	Maximum effect with
dl-Barium pantothenate	1 γ per cc.
Ba salt of *dl*-pantothenic acid diphosphate	Inactive at 70 γ per cc.
Reaction mixture	Equivalent to 5 γ per cc.

The data summarized in the table show that the diphosphate, in common with other esters of pantothenic acid, is biologically inactive. Assays were performed by the bacterial method.[6] The fact that the crude phosphorylated reaction mixture had some activity probably indicates incomplete phosphorylation. However, possible activity of one of the monophosphates or a pyrophosphate which may have been present in the reaction mixture may explain the result. These compounds are under investigation.

[6] Snell, E. E., Strong, F. M., and Peterson, W. H., *J. Am. Chem. Soc.*, 60, 2825 (1938).

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HYPOAMINOACIDEMIA IN CHILDREN WITH NEPHROTIC CRISES

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The failure of children with the nephrotic type of Bright's disease to regenerate plasma protein when fed a diet approximately optimal for the assimilation of nitrogen cannot be attributed solely to loss of protein in the urine in all cases; sometimes severe hypoalbuminemia occurs with but slight loss of urinary protein.[1] Further, rapid regeneration of plasma protein during recovery is, within wide limits, independent of the protein content of the diet.

In observing nephrotic children in this clinic we have also noted that acute febrile episodes with peritoneal symptoms were most frequent in patients with a plasma albumin content below 1 Gm. per hundred cubic centimeters. Sometimes these episodes were accompanied by pneumococcic bacteremia and peritonitis, but often an episode ran its course without such infection. It was suggested in a previous study that when bacterial invasion occurs it may be secondary to acute disturbances in metabolism which cause diminished resistance to infection rather than to a fresh infection.[1c] In favor of this hypothesis was the fact that in many instances the invading type of pneumococcus was recovered from the nasopharynx long before infection of the blood stream or the peritoneum occurred.[2] For the metabolic disturbance and clinical syndrome which seem to occur independently of infection, the term "nephrotic crisis" was suggested earlier.[1c]

We have sought for indications of metabolic changes in these crises and have found the most striking alteration in the concentration of amino acid in the plasma.

Conditions of Observation

All data were obtained on patients in the hospital under continuous observation. Throughout the period of observation there was no improvement in the characteristic

1. Farr, L. E.: (*a*) Assimilation of Protein by Young Children with the Nephrotic Syndrome, Am. J. M. Sc. **195**:70 (Jan.) 1938; (*b*) II. Effect of Dietary Fat and Carbohydrate on Nitrogen Balance, Am. J. Dis. Child. **58**:935 (Nov.) 1939; (*c*) III. Effect of Nephrotic Crises on Assimilation of Nitrogen, ibid. **58**:939 (Nov.) 1939.

2. MacLeod, C. M., and Farr, L. E.: Relation of the Carrier State to Pneumococcus Peritonitis in Young Children with the Nephrotic Syndrome, Proc. Soc. Exper. Biol. & Med. **37**:556 (Dec.) 1937.

nephrotic symptoms of 3 of the patients: G. B., aged 3½ years; J. C., aged 5½ years, and R. F., aged 13 years. One patient, J. T., aged 7 years, was rapidly recovering *from his second attack of nephrosis and had been in the hospital for five months before this work was begun. The fifth patient, R. W., aged 7 years, had just recovered from an attack of acute hemorrhagic Bright's disease in which a marked nephrotic component* had been present. The children were on constant weighed diets throughout the study. During the period of study, none of the children acquired an infection except J. T., who had a minor infection of the upper respiratory tract, from which he made a rapid recovery. J. C., G. B. and R. F. had nephrotic crises. At the time of the first elevation in temperature a blood culture was made, and blood cultures were made frequently during each febrile period. In every instance abdominal paracentesis was performed, and sufficient fluid was withdrawn for culture and study.

Blood for chemical analysis was drawn when the nephrotic crisis had subsided and after the patient had fasted overnight and had gone for at least two hours without fluids. During nephrotic crises the blood was obtained early in the morning but not after an overnight fast, as the patients were fed on a four hour schedule during these episodes. The bleeding was timed to follow a feeding at the maximum interval. To test the ability of these patients to absorb proteolytic products of digestion from the gastrointestinal tract, 2 patients, J. T. and J. C., were fed a single meal with a high protein content after an overnight fast. Each child received in this meal 2 Gm. of protein per kilogram of ideal body weight. J. C. ate 34 Gm. of protein and J. T. 43 Gm. Sufficient fat and carbohydrate were given to insure palatability. Each child required about fifteen minutes to eat the meal. Blood for chemical analysis was obtained during fasting and for three hours after eating, at intervals of forty-five minutes.

After the blood was withdrawn it was transferred with care to a flask containing an oxalate to prevent hemolysis. One portion was immediately centrifuged, and the plasma was removed as completely as possible without disturbing the packed cells. After removal of the plasma the centrifuged cells were laked in enough distilled water to make the volume of cell solution equal to the original volume of whole blood. Aliquots of plasma and cell solution were used for the determinations of amino acid nitrogen, which in most instances were completed within two hours after the withdrawal of the blood. The amounts of nonprotein nitrogen, albumin and globulin in the plasma were also determined. The urea and hemoglobin contents of the portion of whole blood which was not centrifuged were determined.

Methods of Analysis

The amino nitrogen content of the plasma and of the cells was determined by both the Van Slyke nitrous acid method[3] and the MacFadyen and Van Slyke ninhydrin method.[4] The urea nitrogen content of the blood and urine was determined by the hypobromite technic of Van Slyke and Kugel,[5] and the plasma protein and nonprotein

3. Van Slyke, D. D.: Manometric Determination of Primary Amino Nitrogen and Its Application to Blood Analysis, J. Biol. Chem. 83:425 (Aug.) 1929.

4. MacFadyen, D. A., and Van Slyke, D. D.: Determination of Amino Acids in Blood and Urine by Their Reaction with Ninhydrin, to be published.

5. Van Slyke, D. D., and Kugel, V. H.: Improvements in Manometric Micro-Kjeldahl and Blood Urea Methods, J. Biol. Chem. 102:489 (Oct.) 1933.

nitrogen contents were determined by the gasometric micro-Kjeldahl method of Van Slyke.[6] Hemoglobin estimations were carried out gasometrically by the oxygen capacity method of Van Slyke and Neill.[7] Howe's method was used for separation of plasma albumin and globulin.[8] The urea clearances were calculated by the formulas of Møller, McIntosh and Van Slyke,[9] in which the urine values included urinary ammonia. Van Slyke, Page, Hiller and Kirk[10] showed that in cases of nephritis the latter formulas give more consistent clearance values than do those formulas calculated from urinary urea without the ammonia.

RESULTS

The results of the chemical studies of blood in relation to nephrotic crises are given in detail in table 1 and chart 1. They show clearly that nephrotic children are subject to an acute disturbance of plasma amino acid regulation. This disturbance begins without clinical warning, and when not complicated by infection it appears to be self limited. All cultures of blood and ascitic fluid were sterile in the cases of acute illness here reported.

In each instance the acute illness which we have termed "nephrotic crisis" was accompanied by a sharp and sudden fall in the amino acid content of the plasma. We were unable to predict the time of onset of the crises because they began so abruptly. Therefore we were unable to obtain blood immediately before there were any clinical manifestations. We have, however, data on some patients twenty-four to forty-eight hours before the illness developed, and these data showed a plasma amino nitrogen value above 2.5 mg. per hundred cubic centimeters. Blood obtained at the time of the first clinical evidence of illness, namely, the rise in temperature, always showed a severe hypoaminoacidemia which persisted for the period of acute clinical illness, usually forty-eight to fifty-two hours. We obtained blood at the time when the temperature first returned to normal and found that the amino acids had returned to a level above 2.5 mg. of amino acid nitrogen

6. Van Slyke, D. D.: Gasometric Micro-Kjeldahl Determination of Nitrogen, J. Biol. Chem. 81:235 (Jan.) 1927.

7. Van Slyke, D. D., and Neill, J. M.: The Determination of Gases in Blood and Other Solutions by Vacuum Extraction and Manometric Measurement, J. Biol. Chem. 61:523 (Sept.) 1924.

8. Howe, P. E.: The Determination of Proteins in Blood: A Micro Method, J. Biol. Chem. 49:109 (Nov.) 1921.

9. Møller, E.; McIntosh, J. F., and Van Slyke, D. D.: Studies of Urea Excretion: II. Relationship Between Urine Volume and the Rate of Urea Excretion by Normal Adults, J. Clin. Investigation 6:427 (Dec.) 1928.

10. Van Slyke, D. D.; Page, I. H.; Hiller, A., and Kirk, E.: Studies of Urea Excretion: IX. Comparison of Urea Clearances Calculated from the Excretion of Urea, of Urea Plus Ammonia, and of Nitrogen Determinable by Hypobromite, J. Clin. Investigation 14:901 (Nov.) 1935.

TABLE 1

Detailed Data on Blood Chemistry of Nephrotic Children, Showing Relation and Lack of It between Various Plasma Constituents during Nephrotic Crises and on Recovery from Nephrotic Syndrome

Patient, Sex, Age	Date	Plasma Amino Nitrogen (Nitrous Acid Method) Mg. per 100 Cc.	Plasma Amino Nitrogen (Ninhydrin Method) Mg. per 100 Cc.	Plasma Nonprotein Nitrogen, Mg. per 100 Cc.	Whole Blood Urea Nitrogen, Mg. per 100 Cc.	Plasma Albumin, Gm. per 100 Cc.	Plasma Globulin, Gm. per 100 Cc.	Total Plasma Protein, Gm. per 100 Cc.	Hemoglobin, Volume per Cent	Cell Amino Nitrogen (Nitrous Acid Method) Mg. per 100 Cc.	Cell Amino Nitrogen (Ninhydrin Method) Mg. per 100 Cc.	Urea Clearance, per Cent of Normal
J. C.	3/ 1	3.45	2.74	17.0	7.5	0.63	3.23	3.86	20.2	.....	.....	98
♂	3/ 8	4.12	3.09	25.0	12.0	1.41	3.01	4.44	19.5	.....	.....	...
5½ yr.	3/15	3.56	2.80	20.3	10.9	1.22	3.06	4.28	20.8	.....	.....	90
	3/22	3.69	2.92	22.0	8.6	1.28	3.30	4.58	19.0	10.83	6.39	102
	3/24	2.13	2.04	29.0	12.6	1.04	3.16	4.20	19.2	19.87	22.48	122
	3/25	2.58	2.16	25.9	9.9	0.61	3.23	3.84	18.3	15.93	32.99	84
	3/26	2.95	2.10	25.6	8.6	0.36	4.06	4.36	16.8	12.41	19.31	108
	3/28	4.36	3.09	35.5	6.6	0.86	3.65	4.51	16.3	9.84	11.50	...
	4/ 5	3.58	3.09	13.8	10.2	1.14	3.20	4.34	18.1	10.61	10.41	75
	4/12	3.85	3.09	17.9	7.9	1.55	2.63	4.18	18.6	11.15	6.00	83
	4/18	3.87	3.15	12.3	7.6	1.16	2.79	3.95	18.6	11.82	11.45	100
	4/25	3.26	2.74	22.8	10.3	1.08	2.93	4.01	19.4	10.66	.....	66
	5/ 3	2.44	2.27	15.1	9.1	0.81	3.04	3.85	19.2	13.60	.....	61
	5/ 9	3.44	2.57	....	10.0	0.78	3.39	4.17	18.4	14.19	.....	76
	5/10	2.18	2.10	....	8.4	....	....	....	....	10.45	.....	63
	5/11	2.56	2.16	....	15.7	....	....	....	....	11.67	.....	46
	5/12	4.55	1.92	....	6.4	....	....	....	....	10.57	.....	137
	5/13	4.20	3.03	....	19.8	....	....	....	....	7.82	.....	47
	5/17	4.01	....	....	6.5	1.53	3.06	4.59	14.7	12.35	.....	86
	5/23	4.31	3.67	....	11.1	1.76	3.11	4.87	17.0	9.55	.....	77
	5/25	4.32	3.62	....	12.2	....	....	....	17.1	11.12	.....	...
	5/30	3.77	3.27	....	10.6	0.78	2.68	3.46	17.9	11.86	.....	46
	6/ 6	3.92	....	20.5	8.4	1.33	2.85	4.18	17.5	13.32	.....	48
	6/13	4.53	....	22.4	8.7	1.57	2.75	4.32	18.6	14.75	.....	69
	6/20	4.36	....	12.1	8.8	1.43	2.83	4.26	17.4	14.74	.....	67
R. F.	6/10	4.53	2.97	31.0	21.7	1.68	2.36	4.04	....	.....	.....	63
♀	6/11 a.m.	3.76	2.39	35.7	29.3	1.38	2.61	3.99	10.6	18.13	.....	29
13 yr.	6/11 p.m.	3.33	2.57	55.3	36.1	1.42	2.54	3.96	....	19.91	.....	18
	6/12	3.90	2.80	66.3	42.0	1.34	2.82	4.16	11.3	15.53	.....	19
	6/13	3.74	2.97	68.1	46.0	1.53	2.98	4.51	11.3	17.38	.....	20
	6/14	4.88	3.67	....	41.8	....	....	....	....	.....	.....	22
	6/22	5.72	....	35.4	18.8	2.64	2.76	5.40	14.8	17.46	.....	39

TABLE 1—*Concluded*

Patient, Sex, Age	Date	Plasma Amino Nitrogen (Nitrous Acid Method) Mg. per 100 Cc.	Plasma Amino Nitrogen (Ninhydrin Method) Mg. per 100 Cc.	Plasma Nonprotein Nitrogen, Mg. per 100 Cc.	Whole Blood Urea Nitrogen, Mg. per 100 Cc.	Plasma Albumin, Gm. per 100 Cc.	Plasma Globulin, Gm. per 100 Cc.	Total Plasma Protein, Gm. per 100 Cc.	Hemoglobin, Volume per Cent	Cell Amino Nitrogen (Nitrous Acid Method) Mg. per 100 Cc.	Cell Amino Nitrogen (Ninhydrin Method) Mg. per 100 Cc.	Urea Clearance, per Cent of Normal
J. T.	3/ 1	2.97	2.51	20.0	7.5	0.73	3.04	4.07	17.1	.....	.....	132
♂	3/ 8	3.90	2.80	21.0	14.1	1.63	2.82	4.45	16.3	.....	.....	...
7 yr.	3/15	4.48	3.38	17.3	8.0	2.53	2.50	5.03	17.1	.....	.....	105
	3/22	3.40	3.33	16.1	8.7	3.03	2.70	5.73	17.9	11.11	6.06	141
	4/ 5	3.65	3.73	12.2	7.5	2.41	2.35	4.76	17.7	11.00	6.55	86
	4/12	3.80	3.62	21.8	7.3	3.03	2.34	5.37	17.5	11.17	.....	109
	4/18	3.98	3.62	13.5	6.5	2.88	2.08	4.96	17.5	9.29	9.55	130
	4/25	3.70	3.38	18.4	14.9	1.43	2.77	4.20	17.7	10.40	.....	131
	5/ 3	3.58	3.09	18.4	9.7	2.29	2.68	4.97	17.7	10.43	.....	119
	5/ 9	4.08	3.85	....	9.0	2.95	2.28	5.23	18.2	11.53	.....	121
	5/17	4.35	3.91	....	7.9	3.36	2.07	5.43	17.9	11.36	.....	137
	5/23	4.40	3.80	....	8.3	3.29	2.05	5.34	18.0	10.66	.....	119
	5/25	4.49	4.03	....	....	....	....	....	....	11.23	.....	...
	5/30	4.05	3.91	....	9.6	3.61	2.05	5.66	18.7	9.91	.....	140
	6/ 6	4.93	4.03	18.5	8.6	3.47	1.93	5.40	17.9	11.00	.....	145
	6/13	4.41	....	19.5	10.1	3.30	1.99	5.29	17.9	12.53	.....	128
	6/20	4.83	....	18.0	8.1	3.07	2.27	5.34	17.9	12.66	.....	143
G. B.	4/12	3.21	2.16	22.9	....	0.90	2.90	3.80	19.2	15.58	9.87	...
♀	4/14	3.96	2.68	23.5	18.2	1.09	2.83	3.92	16.8	12.66	8.72	49
3½ yr.	4/25	3.69	3.09	25.9	10.0	1.02	3.13	4.15	17.1	16.67	.....	67
	5/ 9	3.90	4.08	....	8.1	1.48	2.94	4.42	17.4	14.18	.....	137
	5/23	4.51	....	....	9.6	0.98	2.76	3.74	17.8	12.08	.....	92
	6/ 6	4.01	....	20.6	8.0	1.25	2.91	4.16	16.7	13.72	.....	94
	6/20	4.66	....	11.0	9.1	0.73	3.09	3.82	15.4	18.81	.....	108
R. W.	4/25	3.65	4.20	22.9	10.8	4.50	2.57	7.07	17.9	10.11	.....	73
♂	5/ 9	3.73	4.15	....	10.2	4.52	2.34	6.86	17.5	12.36	.....	108
7 yr.	5/16	4.14	3.73	....	8.7	4.40	2.47	6.87	17.8	10.28	.....	127

per hundred cubic centimeters of plasma. The sharp onset and critical recovery together with the absence of any prodromal fever are shown by the characteristic temperature curves in chart 2.

Characteristic and constant changes in the urea clearance were lacking. In some instances the clearance was unchanged but in general, with the excessive prostration the clearance decreased, rising again with recovery. The effects on renal function were entirely transitory and of the nature of

the changes seen after fatiguing exercise. The attacks did not appear to affect materially the renal lesion.

In most cases the significant physical findings were limited to the abdomen, the skin and the general appearance. The onset was usually heralded

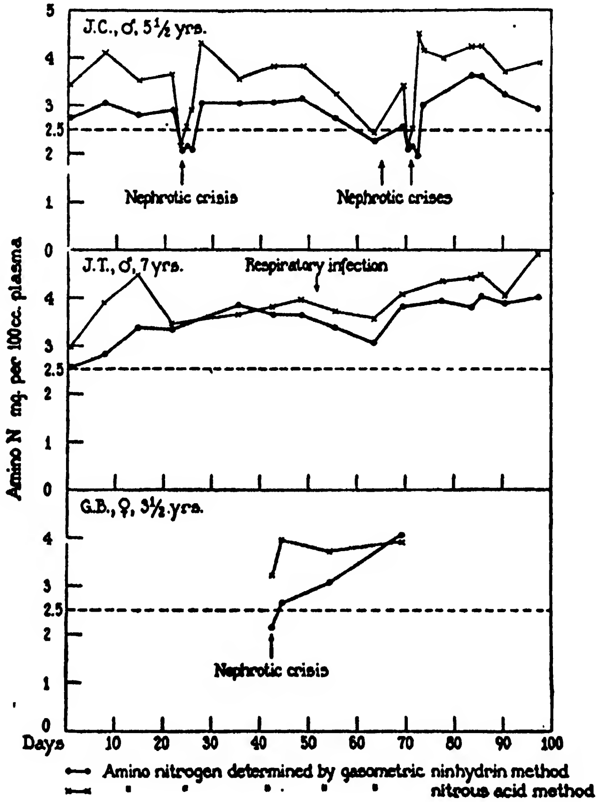

CHART 1. Graphic summary of the plasma amino nitrogen level determined by two methods, showing changes before, during and subsequent to, nephrotic crises in J. C. and G. B. The gradual increase in plasma amino nitrogen during recovery is shown in the graph for J. T. Clinical symptoms are not manifest when the plasma amino nitrogen exceeds 2.5 mg. per hundred cubic centimeters as determined by the ninhydrin method. The normal value by this method probably exceeds 4 mg. per hundred cubic centimeters.

by a complaint of abdominal pain with fever, though rarely fever alone came first. The abdominal pain was general at times but frequently was limited to various regions of the abdomen. Pain in the right lower quadrant of the abdomen was not uncommon. When pain occurred over the kidneys it was severe and became excruciating if the region was palpated or the patient moved. There was no local edema or redness on these occasions. In some

instances the hyperesthesia extended over the inner aspects of the thighs, but there was no erysipeloid reaction. Results of rectal examination were uniformly negative. Gaseous distention was common and frequently approached the severity of paralytic ileus. Abdominal rigidity was lacking unless there was peritoneal infection, but frequently voluntary rigidity was encountered. The rapid, grunting respirations accompanying severe abdominal pain were not associated with any pulmonary involvement. The marked pallor of the child always became exaggerated, and the usual lively interest in the environment was replaced by prostration and listlessness. Cyanosis was observed only with concurrent infection. Nausea was common but vomiting less so; mild diarrhea was noted only occasionally. The

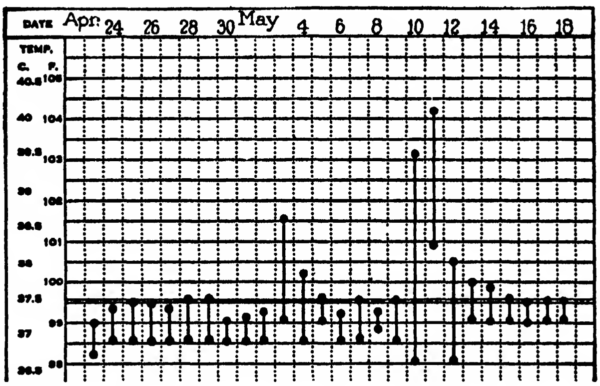

CHART 2. Maximum and minimum daily temperatures of J. C., before, during and after two nephrotic crises with hypoaminoacidemia. Note especially the absence of fever preceding the attacks.

most striking sign of an attack was that the child, usually lively, became suddenly prostrate, listless and pale. By chance, routine physical examination was made in 2 instances about forty-five minutes prior to an attack, and no abdominal tenderness was present, nor were there any other indications of the impending attack.

Recovery is as rapid as onset, and we have seen the temperature fall as much as 5 degrees (F.) in a few hours. A child who has been sick for many hours may suddenly sit up in bed and state that the pain has gone and that he feels well again. Although secondary rises in temperature may occur for another twenty-four hours, the prostration is lacking.

During the attacks we continued to feed the children the same quantity of protein, carbohydrate and fat as previously. However, the diet was made liquid and fed on a four hour schedule. We have frequently given half the day's protein rations as gelatin, which has been well taken by the

patients. Although it does not materially alter the course of the attack, we believe it to be of distinct value, for it furnishes an easily available source of amino acids, and we have the impression that with such a regimen the development of severe paralytic ileus is less common.

The clinical picture of acute illness accompanied a decrease in the plasma amino acids below 2.5 mg. of amino nitrogen per hundred cubic centimeters, as measured by the ninhydrin technic. The severity of the clinical manifestations was roughly proportional to the drop in plasma amino nitrogen below this value. We believe that the changes in the concentration of plasma amino acid occurred as abruptly as the changes in temperature and in the clinical condition of the patient, but it was not practicable to carry out more frequent bleedings on the patients because of the severity of the disturbance. The level of the amino acids in the blood was not apparently influenced by diet during the period of acute deficit in amino acid. One patient, R. F., was given a transfusion of 500 cc. of blood during an attack, with no appreciable improvement either in the plasma amino acid content or in the clinical condition.

In nephrotic children, ordinary infections in the upper respiratory tract, such as the common cold, otitis media, pharyngitis or cervical adenitis with high fever, are not accompanied by this acute metabolic disturbance. It was not cyclic in occurrence and bore no evident relation to any environmental factors.

The acute and characteristic changes noted in the plasma were not accompanied by constant findings in the blood cells. Determination of the cell amino nitrogen content by the technic used included both a red and a white cell count. Leukocytosis was always present during an attack, the white cell counts averaging 18,000 to 20,000. On one occasion (J. C., 3/25) the amount of cell amino acid increased strikingly; usually there was no significant change. The presence of glutathione in the cells, which reacts as an amino acid to both methods, rendered difficult any exact interpretation of the cell values. In addition, because of the difficulty of preparing cell solutions and of the necessity of carrying out their analyses immediately (the necessity does not exist with plasma), we discontinued routine determinations of the cell amino nitrogen content.

A characteristic finding during the attacks was that the nitrous acid method showed an increase in the amino nitrogen twelve to twenty-four hours before the ninhydrin method. The clinical course paralleled the values obtained with the ninhydrin method. The significance of this variation is not known. The nitrous acid method determines all primary amino nitrogen in the blood, including that of any primary amines, amino purines

and peptides that may be present but does not determine proline and oxyproline. The ninhydrin method includes both these amino acids, but excludes other amines and all peptides except those which, like glutathione, have an adjacent NH_2 and COOH group free in a glutamyl or aspartyl group. The specificity of the ninhydrin carboxyl method for the determination of amino acids has been discussed in detail by Van Slyke, Dillon and MacFadyen[11] and by MacFadyen and Van Slyke;[4] it appears to indicate closely the amino acid nitrogen content in analyses of plasma. The importance of using the ninhydrin method was demonstrated in the findings for G. B., who clinically had a characteristic attack in which the amount of plasma nitrogen as determined by the nitrous acid method was well above the critical level, while the ninhydrin method revealed the expected acute deficit.

The sharp decrease in plasma amino nitrogen occurred without significant change in the plasma nonprotein nitrogen or in the urea nitrogen. The changes observed in the plasma albumin and in the hemoglobin were slower in developing and did not parallel the clinical course of the acute illness to the same degree as did the changes in amino acid.

In addition to the sharp decline in amount of plasma amino acids during acute nephrotic crises, there may be a chronic deficit in plasma amino acids throughout the duration of the disease. From this chronic deficit in plasma amino acids there was a definite rise in the plasma amino nitrogen of J. T. during the period of observation; this rise paralleled clinical recovery and improvement in the plasma protein level. With recovery from the nephrotic syndrome the plasma amino nitrogen, as measured by the ninhydrin method, exceeded 4 mg. per hundred cubic centimeters. Unpublished data indicate that this may be the lower limit of normal. J. C. had a continuous amino nitrogen deficit during the entire period of study. The sharp increase in the plasma amino nitrogen of G. B., on the other hand, was not due to permanent recovery but was associated with rapid improvement immediately after her admission to the hospital. Subsequently her plasma amino acid nitrogen values again decreased to chronic deficit levels.

The decrease in plasma amino nitrogen during these crises was not due to failure to digest and absorb dietary protein, since the amount of urinary nitrogen excreted during these periods did not decrease nor was there any significant increase in nitrogen in the stool.[10]

The 2 patients fed a protein meal showed during the three hours after

11. Van Slyke, D. D.; Dillon, R., and MacFadyen, D. A.: Gasometric Determination of Carboxyl Groups in Amino Acids, to be published.

the meal a significant rise in the plasma amino nitrogen value as measured by both methods used. The results are given in table 2. Data on normal children for comparison are not available, but Peters and Van Slyke[12] summarized observations on adults in the conclusion that "In man . . . the maximum rise [in blood amino nitrogen after protein food] appears to be about 2 mg." per hundred cubic centimeters of whole blood. The data in chart 2, obtained by both the nitrous acid and the ninhydrin method,

TABLE 2

Data on Blood Amino Nitrogen, Hemoglobin, Plasma Proteins and Urea after Ingestion of a Protein Meal

Patient	Period	Plasma Amino Nitrogen (Nitrous Acid Method) Mg. per 100 Cc.	Cell Amino Nitrogen (Nitrous Acid Method) Mg. per 100 Cc.	Whole Blood* Amino Nitrogen (Nitrous Acid Method) Mg. per 100 Cc.	Plasma Amino Nitrogen (Ninhydrin Method) Mg. per 100 Cc.	Cell† Amino Nitrogen (Ninhydrin Method) Mg. per 100 Cc.	Whole Blood Amino Nitrogen (Ninhydrin Method) Mg. per 100 Cc.	Plasma Ratio Amino Nitrogen (Nitrous Acid Method) to Amino Nitrogen (Ninhydrin Method)	Oxygen Capacity, Vol. per Cent	Total Plasma Protein, Gm. per 100 Cc.	Plasma Urea Nitrogen, Mg. per 100 Cc.
J. C.	Fasting . . .	3.69	10.78	6.63	3.08	6.20	4.39	1.20	19.0	4.58	8.6
	45 min.. . .	4.68	10.57	7.18	4.15	6.80	5.30	1.13	19.3	4.31	9.9
	90 min. .	4.86	11.39	7.83	4.69	5.40	5.14	1.04	20.0	4.40	11.8
	135 min.....	5.39	11.66	8.05	4.69	6.80	5.62	1.15	19.2	4.29	11.1
	180 min.....	5.30	11.60	8.18	5.14	7.39	6.24	1.03	19.8	4.38	12.7
J. T.	Fasting . . .	3.39	11.10	6.31	3.48	6.16	4.36	0.97	17.9	5.73	8.7
	45 min.....	5.06	12.62	7.63	5.24	6.96	5.67	0.97	16.9	5.33	12.1
	90 min.....	4.38	10.00	6.41	5.66	6.10	5.71	0.77	17.6	5.31	14.7
	135 min...	4.51	11.93	7.18	5.60	5.07	5.26	0.81	17.4	5.67	13.0
	180 min.....	4.83	10.73	7.06	7.10	6.29	6.70	0.68	18.0	5.72	14.8

* By addition.

† Analyses done on tungstic acid filtrate.

indicate increases of blood amino nitrogen of similar magnitude. Furthermore, an increase of 1 mg. or more in the plasma amino nitrogen occurred in the first forty-five minutes after the meal. Evidently digestion, as well as absorption of the amino acids produced, began without delay.

A further point of general interest is the determination of the amino acid nitrogen content of the cells after the protein meal did not show changes which paralleled the changes in the plasma. The variations in cell amino acid content, ranging in the three hours over ± 5 per cent of the median

12. Peters, J. P., and Van Slyke, D. D.: Quantitative Clinical Chemistry, Baltimore, Williams & Wilkins Company, 1931, vol. 1, p. 389.

value in each case, showed no regular relation to the process of protein digestion. The normally constant values obtained for hemoglobin, plasma albumin and total protein indicate that no serious disturbance of blood volume followed the withdrawal of samples for analysis.

An interesting difference between the data for the 2 patients who had eaten a protein meal was noted in the ratio of amino nitrogen determined by the nitrous acid method to amino nitrogen determined by the carboxyl method. For J. C. this ratio was never less than 1 and ranged from 1.20 during fasting to 1.03 three hours after eating. For J. T., on the other hand, the ratio never exceeded 1 and ranged from 0.97 during fasting to 0.68 after three hours. At present we have no information on the significance of these ratios.

COMMENT

Previous studies by other workers[13] of blood amino acids in nephritic and nephrotic patients have not revealed either the acute or the chronic type of disturbance. Many of the observations were made with the Folin colorimetric method which Van Slyke and Kirk[14] showed to be unreliable. In addition, most of the analyses were done on whole blood, in which the change would be difficult to detect because the amino nitrogen content of the cells is much greater than that of the plasma. Kirk[13] has reported from the clinic of the Rockefeller Institute work on amino nitrogen of plasma and of whole blood of nephritic and nephrotic patients. He used the Van Slyke gasometric nitrous acid method. Kirk confirmed the reports of previous workers that an increase in primary amino nitrogen may occur in adult nephritic patients, particularly those with severe renal failure. Kirk's youngest patient was 15 years old and had renal failure at the time of the study. It seems most likely that the contrast between the results obtained in studies of nephritis by previous workers and those in our studies is due chiefly to a difference in the type of patient studied. We have also carried out a few analyses on adults having nephrosis and to date have been able to substantiate Kirk's findings in cases of moderate to severe impairment of renal function. In an adult with nephrosis and a nearly normal urea clearance, we noted the same type of chronic deficit in plasma amino acid which has been here described as occurring in children. The acute disturb-

13. Cited by Kirk, E.: Amino Nitrogen Changes of the Blood in Nephritis, J. Clin. Investigation **12**:1091 (Nov.) 1933.

14. Van Slyke, D. D., and Kirk, E.: Comparison of Gasometric, Colorimetric and Titrimetric Determinations of Amino Nitrogen in Blood and Urine, J. Biol. Chem. **102**:651 (Oct.) 1933.

ance of plasma amino nitrogen has thus far in our experience been limited to young children with the nephrotic syndrome.

SUMMARY

Two hitherto unknown disturbances of the amino acids in children with the nephrotic type of Bright's disease are described.

A chronic deficit in plasma amino acids often accompanies the disease. The content of plasma amino acid nitrogen is between 3 and 4 mg. per hundred cubic centimeters, in comparison with the usual normal content of 5 mg. or over. Recovery is accompanied by a rise in the plasma amino nitrogen to normal values.

An acute decline in plasma amino acid nitrogen to below 2.5 mg. per hundred cubic centimeters and sometimes to nearly 1 mg. per hundred cubic centimeters occurs uniformly with the clinical nephrotic crises described. The crisis occurs without infection. The acute clinical manifestations become apparent when the amino acid nitrogen drops below the critical level of 2.5 mg. per hundred cubic centimeters of plasma. Recovery from acute symptoms and return of plasma amino acid nitrogen to its previous level occur together. Recovery may be as rapid as the onset and take but a few hours.

The postprandial increase in plasma amino acid nitrogen after a heavy protein meal in the cases of 2 children with the nephrotic syndrome indicates no delay in protein digestion or absorption.

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THE INTRAVENOUS ADMINISTRATION OF SMALL DOSES OF CASEIN HYDROLYSATE TO NEPHROTIC CHILDREN AND ITS EFFECT UPON THE NITROGEN BALANCE AND PLASMA AMINO ACID LEVEL

BY LEE E. FARR, M.D.

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Recently Farr and MacFadyen[1] reported finding a chronic plasma hypo-aminoacidemia in nephrotic children and an acute exacerbation of this plasma amino acid deficit during nephrotic crises. Failure to control the plasma amino acid deficit on diets which promote optimal nitrogen assimilation in nephrotic children[2] led to the consideration of the therapeutic use of amino acid mixtures. Van Slyke and Meyer[3] showed that moderate amounts of amino acids given to dogs intravenously were removed rapidly from the blood stream and there were no apparent toxic effects. Because of the difficulties of preparing a satisfactory digest for human use, there was little further work reported on this problem until recently. In 1937, Elman[4] reported studies showing the practicability of casein digests as a source of nutritional nitrogen. Elman gave the digest intravenously and reported no severe reactions. Farr and MacFadyen[5] have reported further studies on nephrotic children which show that amino acids given intravenously are not immediately lost in the urine. This demonstrated the feasibility of this procedure for providing an extra source of amino acids in these patients. The present study was carried out to determine the effects upon the level of the plasma amino acids and upon the nitrogen balance of intravenous administration of a casein digest to children with the nephrotic syndrome.

Materials and Methods

The nitrogen content of the diets was calculated from standard tables on food analysis. Throughout the period of study the same weekly diet was used. This permitted some variation in the daily menu and provided for a check on any inaccuracies of the calculated diet since the patients received the same foodstuffs during periods of amino acid administration and rest periods.

Read in abstract before the Society for Pediatric Research, Skytop, Pa., April 27, 1939.

The urine samples were collected in clean sterile bottles, and whenever practicable, the patient voided directly into the bottle. No preservative was used. At the end of each twelve-hour period, each bottle was tightly stoppered and stored in the icebox until the end of the metabolic interval of study. At this time the entire urine specimen was thoroughly mixed, and aliquots were removed for analysis. Stool specimens were collected in clean pots, sent to the laboratory immediately after they were obtained, and transferred quantitatively to a concentrated sulfuric acid mixture. At the end of each period, the acid stool mixture was thoroughly mixed, made up to a convenient volume, and aliquots were removed for analysis. The beginning of each period was marked by feeding the patients 0.2 Gm. of carmine with breakfast, and the first stool containing carmine was taken as the first specimen of the ensuing period. No laxatives were necessary at any time.

Standard macro-Kjeldahl procedures were used for determining total stool nitrogen and urine nonprotein nitrogen after deproteinization with trichloracetic acid. Urine protein nitrogen was determined by a slight modification of the biuret colorimetric procedure of Hiller.[6] Urea plus ammonia nitrogen in the urine was determined by the method of Van Slyke and Kugel.[7] Plasma amino acid nitrogen was determined by the ninhydrin-CO_2 method of MacFadyen and Van Slyke.[8] The other clinical chemical analyses were carried out by the methods routinely used in this laboratory.[9-14]

The casein digest* used was an enzymatic product, required no additional amino acids, and satisfied the nutritional needs of rats.[15] The dry powder, packed in vacuum sealed cans, was kept unopened in the icebox until needed. The dry powder was weighed and dissolved in hot fresh triple distilled water at about 100°C. At this temperature solution was readily effected. Enough powder was used to give a solution containing approximately 10 per cent casein hydrolysate by weight and to yield lots of about 5 liters of the final solution. After solution was apparently complete, the material was kept on the steam bath for from thirty minutes to one hour, filtered through a Seitz filter while hot, bottled aseptically in lots of 100 c.c., autoclaved and stored in the icebox until used. The final solution had a reddish chestnut-brown color. The pH of the final product determined with a glass electrode averaged 4.45. No buffers were added. Material prepared in this way has been used after six months in the icebox with no reactions in the patient following its use. In some bottles a flocculent

* Obtained through the generosity of Mead Johnson and Company.

black precipitate formed after a few days in the icebox. This was removed before use by filtering through a sterile gauze square. Prior to clinical use, an aliquot of each lot was tested in rabbits for chill-producing substance.† If this test was satisfactory, the solution was ready for clinical trial.

Clinical Course of Patients during Study and Experimental Procedures

Four patients on the wards of the hospital were selected for study, two males, J. C., aged 6 years, and W. O'B., aged 4 years, and two females, R. Q., aged 7 years, and E. S., aged 7 years. All had been under observation on the hospital wards for several months prior to beginning this study. All had a history of insidious onset of edema without antecedent infection. At the time this study was begun, all had characteristically low plasma proteins, normal hemoglobin, normal blood urea nitrogen, normal blood pressure, normal urea clearance, and absence of hematuria. They were free from obvious acute or focal infection and had no clinically demonstrable edema or ascites. Previously, for three months, nitrogen balance studies had been carried out on three of the patients who had been on weighed calculated diets of approximately the same content as used in this study. One patient, E. S., had been on an approximate unweighed diet of the same proportions until two weeks prior to beginning this study, when she was started on the weighed and calculated diet used in these observations. Laboratory and clinical findings on these patients are summarized in Figs. 1 to 4. The present study was carried out on J. C. during months twenty-five to twenty-nine, on W. O'B. during months five to nine, on R. Q. during months six to nine, on E. S. during months fifty to fifty-four.

The four children were observed on a nitrogen balance study for fifteen consecutive weeks. A résumé of the diets used during the period of observation is given in Table I. Two one-week periods of study were completed before any amino acids were given intravenously. During the third period, 10 per cent casein hydrolysate solution mixed with an equal volume of 10 per cent glucose was given before breakfast on the first, third, and fifth days. The solution was allowed to run into the vein by gravity. A 20 gauge needle was used, and the bottom of the infusion flask was about 20 inches above the vein. At the conclusion of each amino acid infusion, a few cubic centimeters of 5 or 10 per cent glucose were run in to insure complete administration of the digest. The same technique of administra-

† I am indebted to Dr. Kenneth Goodner who carried out these tests in his laboratory.

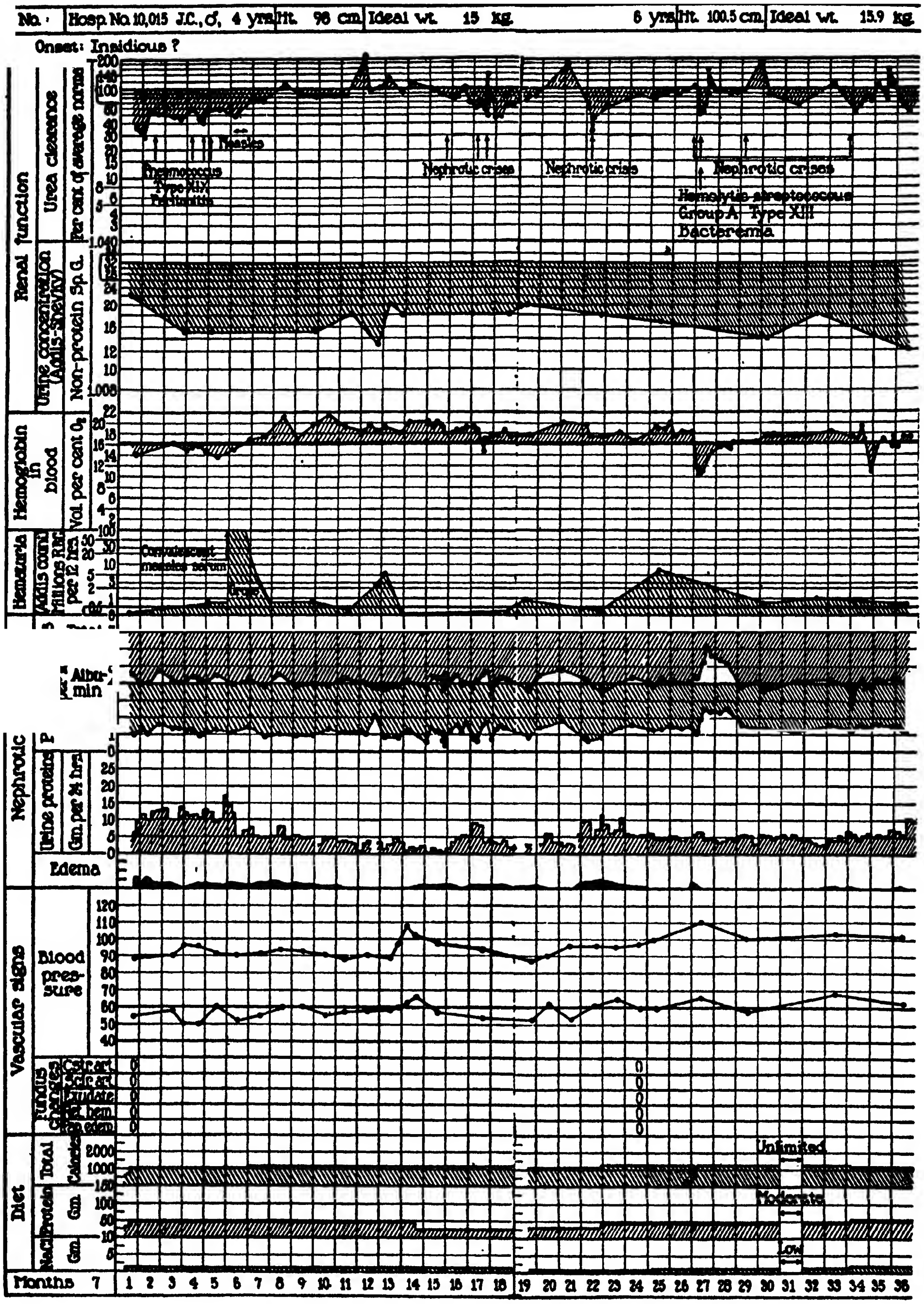

Fig. 1. Graphic summary of course of J. C. since admission to the hospital. The present study covers months twenty-five through twenty-eight. Note the absence of effect upon renal function following the hemolytic streptococcus infection in month twenty-seven.

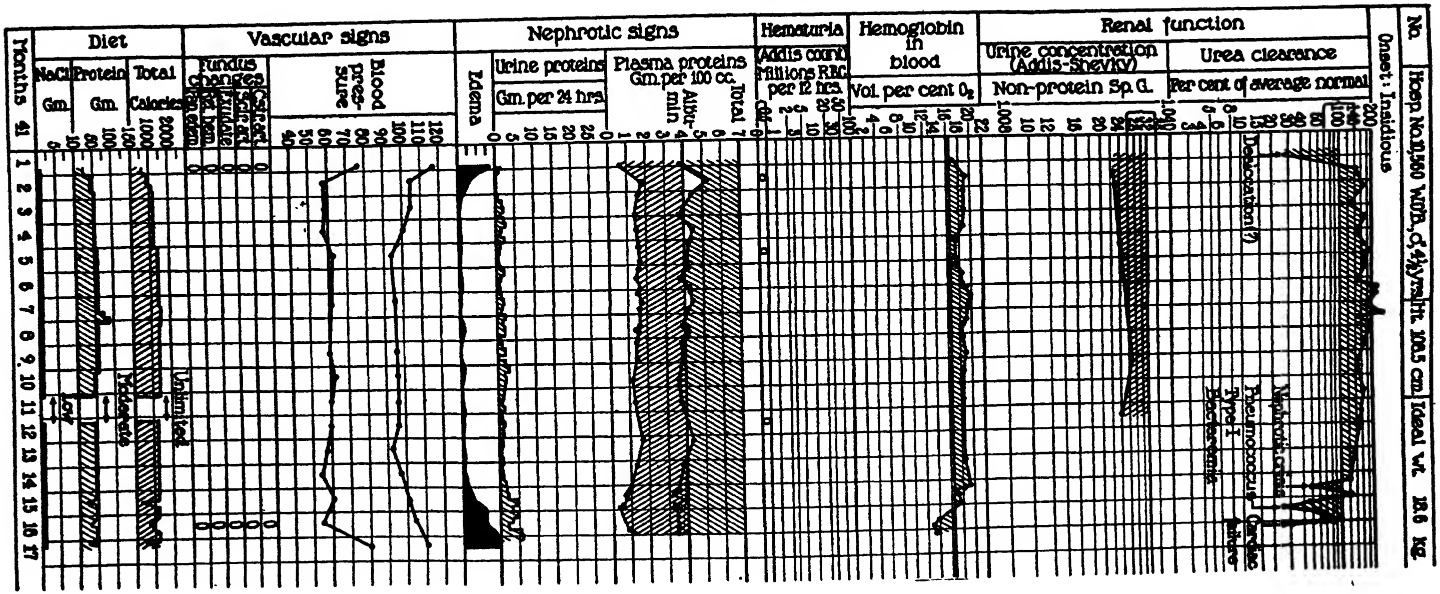

FIG. 2. Graphic summary of course of W. O'B. since admission to the hospital. The present study covers months five to eight.

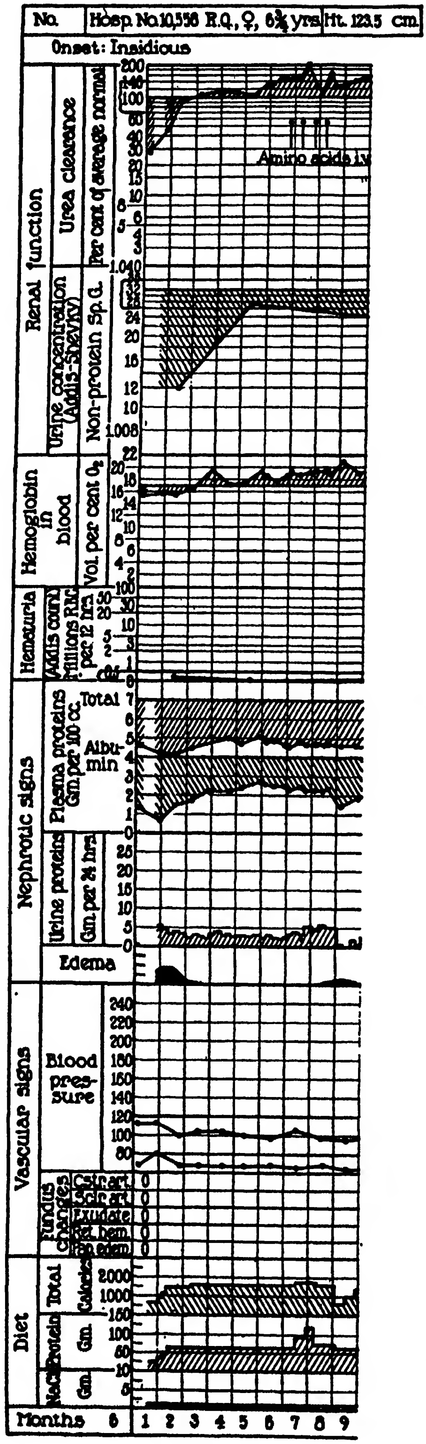

FIG. 3. Graphic summary of course of R. Q. since admission to the hospital. The present study covers months six through nine.

tion was used during all subsequent periods. No amino acids or glucose were given on the second, fourth, sixth, and seventh days. The quantity

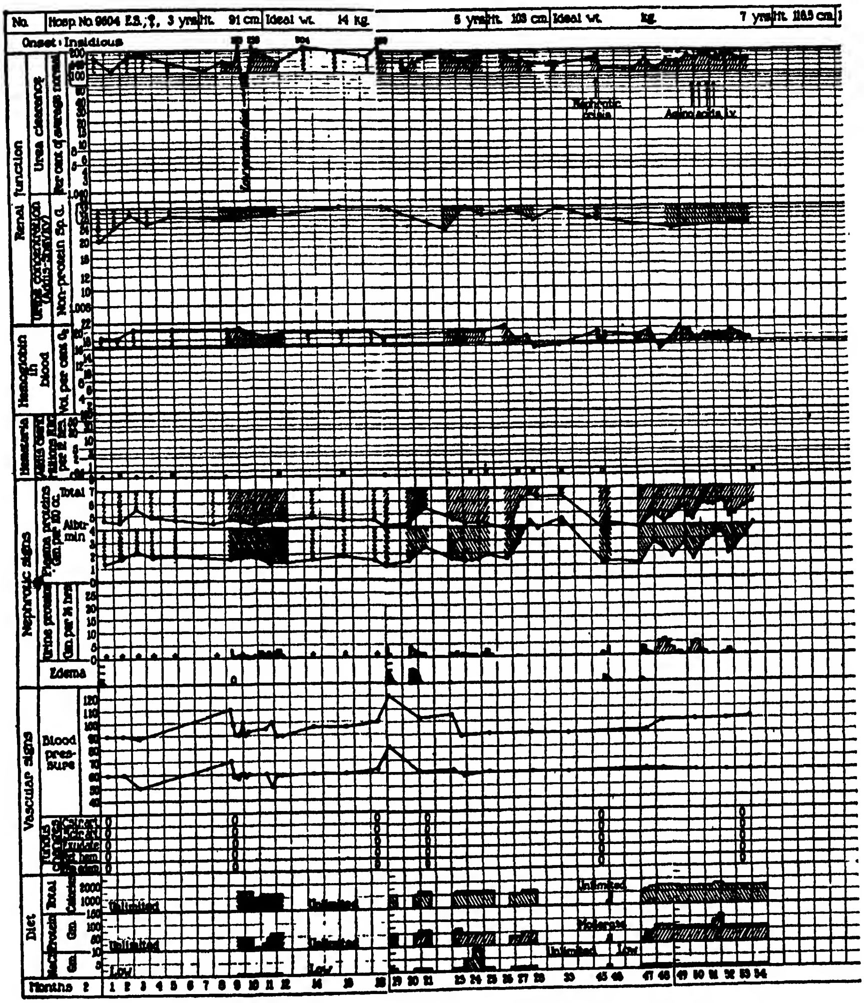

FIG. 4. Graphic summary of course of E. S. since admission to the hospital. The present study covers months fifty through fifty-three. This child made a complete recovery from her first attack in the twenty-eighth month and was well until three weeks before readmission in the forty-fifth month. She has made a complete recovery from her second attack and now has been well for ten months.

given to each patient varied, since the infusion was discontinued promptly when the patient became nauseated. W. O'B. was given 10, 2.7, and 5 Gm. of the amino acids; J. C. was given 5, 2, and 5 Gm.; R. Q. was given

10, 2.5, and 5 Gm.; E. S. was given 5, 2.6, and 5 Gm. A second control study of three one-week periods followed. During the seventh period, 5 Gm. of casein hydrolysate in the 10 per cent solution were given each morning before breakfast for five successive days to each of the four patients. Glucose was not administered simultaneously during this period. The first day of the seventh period J. C. developed a fever of 101° F. which returned to normal in three hours after amino acids had been given. He complained of a scratchy throat. Physical examination was negative, except for a slight reddening of the throat. He had no more fever during this period. The first day of the eighth period, he was given no amino acids intravenously. He developed a fever of 103° F. and a sore throat, but did not appear to be gravely ill. His temperature returned rapidly to normal after taking some aspirin and remained normal all night. Blood culture was sterile. The second day of this period his temperature rose rapidly to 105° F., and at noon the patient was prostrated with a grave illness. While his temperature was rising to a peak of 108° F. by 1:30 P.M., a blood culture was taken, and another at the temperature peak. At the height of his temperature an infusion of 125 c.c. of 25 per cent glucose was given with 1 Gm. of aspirin, followed by a tepid sponge. Within two hours his temperature fell to 103.6° F., and the patient developed a generalized convulsion. Though no information from the blood cultures was available, on clinical grounds the infection appeared to be of hemolytic streptococcus origin, and late in the afternoon neoprontosil intramuscularly was begun. The 2.5 per cent solution was used; 5 c.c. were given every four hours for two doses, and 2.5 c.c. every four hours thereafter. On this day he also received two additional infusions of 20 per cent glucose of 125 c.c. each. The following morning the patient's two blood cultures taken the previous day were reported as positive for hemolytic streptococcus Group A, Type 13 (Lancefield). The first culture contained about 5 colonies per cubic centimeter and the second about 200 colonies per cubic centimeter. Although by the morning of the third day the patient's temperature was down to 102° F., he remained gravely ill. Blood chemistry studies showed a plasma amino nitrogen of only 2.10 mg. per cent; therefore, 10 Gm. of amino acids in 10 per cent solution were given intravenously in addition to 28 Gm. of glucose in 10 per cent solution and 40 c.c. of convalescent human scarlet fever serum. Later in the day, on two occasions, the patient was given an additional 40 c.c. of convalescent scarlet fever serum. The neoprontosil therapy did not result in a satisfactory concentration in the blood, the value being below 1 mg. per cent after twenty-four hours, so the patient was changed on this evening to

sulfanilamide by mouth, 0.66 Gm. every four hours for two doses and then 0.33 Gm. every eight hours. The morning of the fourth day his temperature was nearly normal, the blood sulfanilamide concentration was 7 mg. per cent, and the plasma amino acid nitrogen, 2.09 mg. per cent. Ten grams of amino acids in 10 per cent solution, plus 25 Gm. of glucose in 10 per cent solution and 40 c.c. of convalescent serum, were given intravenously. An attempt was made to match the patient for transfusion, but no suitable donor could be found. The patient's blood agglutinated several donors of the same type. His temperature fell gradually to normal, and two days later sulfanilamide therapy was discontinued. Further convalescence was uneventful. During his acute illness the patient was fed a liquid diet with the same caloric, protein, carbohydrate, and fat content as his regular diet. Except for two days when he was critically ill, this diet was surprisingly well taken. This was made possible by the careful nursing care by Miss E. Glantz and the nurses under her, and the skillful

TABLE I

Summary of Diets Fed during Balance Experiment Showing Amounts of Protein, Fat, and Carbohydrate Fed Daily during Each Period

Patient	Ideal weight (kg.)	Actual weight (kg.)	Periods	Average daily food intake								Percentage of calories from		
				Protein (gm.)	Fat (gm.)	Carbohydrate (gm.)	Calories	Protein (gm./kg.)	Fat (gm./kg.)	Carbohydrate (gm./kg.)	Calories (per kg.)	Protein	Fat	Carbohydrate
J. C.*	17.2	18.0	1–16	45	45	171	1,270	2.6	2.6	9.9	74	14	32	54
R. Q.	24.4	21.2	1– 7	67	67	196	1,650	2.8	2.8	8.0	68	19	42	47
			8– 9	98	67	194	1,775	4.0	2.8	8.0	73	22	34	44
			10–11	122	80	142	1,778	5.0	3.3	5.8	73	27	41	32
			12–16	73	73	175	1,650	3.0	3.0	7.2	67	18	40	42
W. O'B.	17.7	16.0	1– 7	56	56	168	1,395	3.2	3.2	9.5	79	17	36	47
			8– 9	71	56	167	1,458	4.0	3.2	9.4	82	20	34	46
			10–11	89	66	127	1,457	5.0	3.7	7.2	82	24	41	35
			12–16	53	53	177	1,395	3.0	3.0	10.0	79	15	34	51
E. S.	21.3	23.4	1– 7	64	64	150	1,433	3.0	3.0	7.0	67	18	40	42
			8– 9	85	64	150	1,516	4.0	3.0	7.0	71	22	38	40
			10–11	107	74	105	1,516	5.0	3.5	4.9	71	28	44	28
			12–16	64	64	150	1,433	3.0	3.0	7.0	67	18	40	42

* Diet taken incompletely during acute infection in period 8.

compounding of the diet by Miss M. Rhine and her staff in the diet kitchen. During the remainder of the study the patient had no further illnesses.

Beginning with period eight, the diet of E. S., R. Q., and W. O'B. was increased to 4 Gm. of protein per kilogram of ideal body weight. The patients were kept on this diet for two one-week periods. The protein content of the diets was then increased to 5 Gm. per kilogram for two periods, the first of four days and the second of three days. After this time on the very high diet, the protein content was reduced to 3 Gm. per kilogram where it was maintained for the duration of the study. Because of his severe illness, the diet of J. C. was not increased during these periods. Following restoration of optimal protein diets, a control period of one week elapsed before amino acid administration was resumed. During period 14, 5 Gm. of casein hydrolysate as a 10 per cent solution was given intravenously before breakfast daily for five days. The amino acid solution was mixed with an equal volume of 10 per cent glucose during this period and also whenever administered during period fifteen. During period fifteen, W. O'B. and R. Q. received 5 Gm. each on the fourth and sixth days of the period; E. S. received 10 Gm. on each of these days, and J. C., 15 Gm. on the fourth and 10 Gm. on the sixth day. During the final week another control study was run.

Throughout the study the urea plus ammonia nitrogen of the urine was determined, and its ratio to the nonprotein nitrogen of the urine was calculated.

Except for the above-described illness of J. C., all the children were free from infection and fever throughout the period of study. Fortunately, there was no vomiting of food during the balance study except on a very few occasions, and an equivalent amount of food was immediately re-fed. No corrections in nitrogen balance were necessary for accumulation or loss of edema or elevation of blood nonprotein nitrogen during the study.

RESULTS

No severe reactions followed the intravenous administration of the casein hydrolysate. The patients all developed a marked flush at the beginning of the infusion which persisted throughout the time the injection was being given and for about fifteen to twenty minutes after its conclusion. A sensation of warmth was noted at the same time by the patients. These effects were not altered by the speed of injection. On numerous occasions a sensation of nausea was complained of, and in several instances this was followed by rather severe retching. In these patients, the development of nausea and vomiting seemed to be related not only to the

rate of injection but also to the total amount given. With small doses, e.g., 5 Gm., given slowly, i.e., during thirty minutes, there was little nausea and no vomiting. When this amount was injected within a period of from five to seven minutes, nausea usually developed, but emesis did not always follow. When 10 Gm. or more were given in a twenty-minute period, nausea was common, and frequently there was retching. One patient, W. O'B., always developed retching whenever the total amount administered at any single injection exceeded 6 Gm. This reaction could not be avoided in this patient by very slow injection. J. C., on the other hand, was able to take 15 Gm. at one administration without vomiting, although there was very definite nausea. When the material was given after meals, both of these reactions were exaggerated to such an extent that administration under these conditions had to be stopped. During the period of amino acid injection, the patients felt unusually well and played with great vigor throughout the day. Their appetites remained excellent.

The rise in plasma amino acid nitrogen immediately after injection was quite constant. After 5 Gm. was given on each of two occasions, the plasma amino acid nitrogen for E. S. rose in one instance from 3.47 to 8.28 mg. per cent and on the other occasion from 3.63 to 6.65 mg. per cent. In the latter instance, thirty minutes later the value had dropped to 4.09 mg. per cent. The administration of a like amount to J. C. on each of two occasions was followed by a rise from 3.14 to 8.72 mg. per cent and from 3.30 to 7.83 mg. per cent, which in thirty minutes had decreased to 4.23 mg. per cent. After 10 Gm., the plasma amino acid nitrogen of R. Q. rose from 3.47 to 12.07 mg. per cent, and in W. O'B., from 3.33 to 11.61 mg. per cent. There were no significant changes in the concentration of the blood urea nitrogen nor increased urea excretion in the urine immediately following the injections. Subsequent studies, as yet unpublished, indicate that, within one hour after administration of amino acids intravenously, the blood level has returned to its original value. Although the injected amino acids were retained, there was no decrease in the plasma amino deficit following a series of injections. This absence of effect on the fasting level is shown in Figs. 5 to 8. After a series of injections, the fasting level was somewhat lower on W. O'B. in periods 3 and 14, on E. S. in period 15, on R. Q. in periods 3, 7, and 15. There was a slight rise on W. O'B. in periods 7 and 15, on E. S. in periods 3, 7, and 14, and on J. C. in period 3. Significant but transient rises were seen on J. C. in periods 7 and 15, and in R. Q. in period 14. The variation seen in these periods was no greater than spontaneous variations previously observed in these same patients.

TABLE II

Summary of Nitrogen Balance Data on Four Patients to Whom the Casein Hydrolysate Was Given Intravenously As a 10 Per Cent Solution

Patient	Period	Number of days	Daily average: Dietary nitrogen intake (gm.) A	Casein hydrolysate nitrogen (gm.) B	Urine nonprotein nitrogen (gm.) C	Urine protein nitrogen (gm.) D	Stool nitrogen (gm.) E	Balance (gm.) (A + B) − (C+D+E)	Ratio in urine of $\frac{\text{Urea N} + \text{NH}_3\text{ N}}{\text{Nonprotein N}}$
J. C.	1	7	7.200		5.330	0.736	1.086	0.048	—
	2	7	7.200		5.150	0.768	1.125	0.157	0.86
	3	7	7.200	0.219[1]	5.248	0.832	1.211	0.128	0.86
	4	7	7.200		5.303	0.736	1.126	0.035	0.84
	5	7	7.200		5.317	0.624	1.077	0.182	—
	6	7	7.200		5.405	0.784	1.291	−0.272	0.87
	7	5	7.200	0.640[2]	5.899	1.008	1.094	−0.161	0.85
	8	9	6.364	0.284[1]	4.803	1.104	1.108	−0.182[3]	0.81
	9	7	7.200		5.264	0.640	1.160	0.136	—
	10	7	7.200		4.496	0.688	1.162	0.854	—
	11–12	7	7.200		4.935	0.608	1.206	0.451	0.86
	13	7	7.200		4.795	0.544	0.996	0.865	0.88
	14	5	7.200	0.640[1]	5.040	0.672	0.987	1.141	0.85
	15	9	7.200	0.333[4]	5 307	0.800	1.049	0.377	0.86
	16	7	7.200		5.307	0.672	1.006	0.215	0.88
W. O'B.	1	7	8.960		7.678	0.112	1.247	−0.077	—
	2	7	8.960		7.568	0.224	1.056	0.112	0.89
	3	7	8.960	0.324[1]	6.972	0.272	1.090	0.950	0.94
	4	7	8.960		7.249	0.384	1.069	0.258	0.86
	5	7	8.960		7.089	0.224	1.085	0.562	—
	6	7	8.960		7.467	0.132	1.014	0.347	0.89
	7	5	8.960	0.640[2]	8.043	0.163	1.110	0.284	0.88
	8	9	8.960		7.575	0.256	1.038	0.091	0.89
	9	7	11.360		9.421	0.384	1.068	0.487	—
	10	7	11.360		9.399	0.312	1.434	0.215	—
	11	4	14.240		11.586	0.288	1.159	1.207	0.91
	12	3	14.240		12.062	0.400	1.228	0.550	0.90
	13	7	8.480		7.679	0.288	0.998	−0.485	0.89
	14	5	8.480	0.640[1]	7.362	0.430	1.253	0.075	0.89
	15	9	8.480	0.133[4]	7.493	0.304	0.984	−0.168	0.89
	16	7	8.480		6.956	0.240	0.980	0.304	0.89

[1] Equal volume of 10 per cent glucose given simultaneously.

[2] No glucose administered simultaneously.

[3] Total nitrogen intake includes 1.665 Gm. of nitrogen in convalescent serum given during acute infection with hemolytic streptococcus.

[4] Given with glucose one hour after meals.

TABLE II—*Concluded*

Patient	Period	Number of days	Daily average: Dietary nitrogen intake (gm.) A	Casein hydrolysate nitrogen (gm.) B	Urine nonprotein nitrogen (gm.) C	Urine protein nitrogen (gm.) D	Stool nitrogen (gm.) E	Balance (gm.) (A + B) − (C+D+E)	Ratio in urine of (Urea N + NH₃ N) / Nonprotein N
R. Q.	1	7	10.720		8.251	0.464	1.151	0.854	
	2	7	10.720		8.414	0.576	1.347	0.383	0.93
	3	7	10.720	0.320[1]	8.654	0.432	1.080	0.880	0.90
	4	7	10.720		8.465	0.592	1.224	0.439	0.86
	5	7	10.720		8.672	0.426	1.116	0.506	
	6	7	10.720		8.811	0.416	1.070	0.423	0.85
	7	5	10.720	0.640[2]	9.031	0.464	1.293	0.572	0.89
	8	9	10.720		8.504	0.576	1.464	0.176	0.91
	9	7	15.680		12.036	0.688	1.535	1.421	
	10	7	15.680		13.046	0.592	1 648	0.394	
	11	4	19.520		15.517	0.832	1.677	1.494	0.92
	12	3	19.520		15.616	0.816	1.945	1.143	0.91
	13	7	11 680		9.672	0.704	1.414	−0.110	0.90
	14	5	11 680	0 640[1]	9.907	1.003	1.305	0.105	0.89
	15	9	11.680	0.133[1]	9.568	0.800	1.280	0.165	0.88
	16	7	11.680		9.048	0.784	1.146	0.702	0.90
E. S.	1	7	10.240		8.602	0.181	1.275	0.182	
	2	7	10.240		8.518	0.096	1.439	0.187	0.88
	3	7	10.240	0 230[1]	8.913	0.288	0.921	0.349	0.88
	4	7	10.240		8.298	0.688	1.345	−0.091	0.86
	5	7	10.240		8.073	0.864	1.390	−0.087	
	6	7	10.240		7.908	0.336	1.304	0.692	0.88
	7	5	10.240	0.640[2]	8.183	0.139	1.111	1.492	0.88
	8	9	10.240		8.591	0.000	1.167	0.482	0.89
	9	7	13.600		10.737	0 000	1.564	1.299	0.93
	10	7	13.600		11.652	0 040	1.337	0.571	
	11	4	17.120		14.447	0.000	1.411	1.262	0.90
	12	3	17.120		14.796	0.030	1.288	1.006	0.90
	13	7	10.240		8.888	0.136	1.242	−0.026	0.89
	14	5	10.240	0.640[1]	8.661	0.239	1.177	0.803	0.89
	15	9	10.240	0.267[4]	8.546	0.432	1.271	0.258	0.88
	16	7	10.240		8.093	0.142	1.160	0.845	0.90

The pigment responsible for the color of the amino acid solution appeared to be immediately and quantitatively excreted in the urine. After an injection, the color of the urine closely approached that of the solution administered, and although we did no careful studies on this pigment, the earlier study on urine nitrogen after amino acid injection indicated that no significant loss of injected nitrogen occurred.

The effects on the nitrogen balance are shown in Table II and Figs. 5

to 8. R. Q. was on a positive nitrogen balance in every period except 13. This period began a lower protein diet than was given in period 12. The excess loss of nitrogen during this period was apparently due to the fact that the patient was not able to attain immediate equilibrium with the

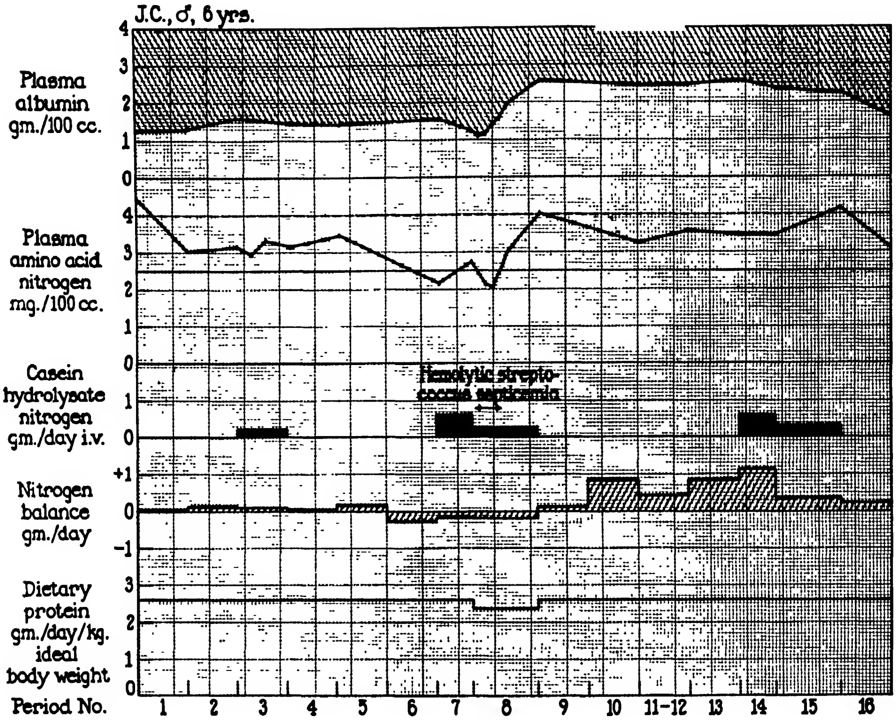

FIG. 5. Summary of nitrogen balance studies on J. C. Note the negative nitrogen balance in period 6, one full week before the first nephrotic crisis. No effects upon the level of plasma amino acids followed injection of casein hydrolysate. Plasma albumin rose paradoxically during period 8, when the patient was on a negative nitrogen balance, and fell through periods 15 and 16, when nitrogen balance was positive. Normal average plasma amino acid nitrogen 4.50 mg. per 100 c.c.

lowered protein intake. The small amount of nitrogen lost for the period, however, indicated that she was able to make a fairly rapid adjustment to the diminished intake of nitrogen. On the other hand, the very large positive balances noted in periods 9 and 11 were caused by an increase in dietary protein without allowing an interval to elapse for attaining equilibrium on the higher diet. The maintenance of a positive nitrogen balance during periods 10 and 12 and failure to excrete an amount of nitrogen during period 13 equivalent to the excess retained during the adjustment periods

9 and 11, indicated that apparently all of the nitrogen retained during the attainment of nitrogen equilibrium on the changed diets was actually assimilated. The administration of amino acids to this patient did not markedly alter the nitrogen balance. W. O'B. was on a positive nitrogen balance throughout the study except for periods 1, 13, and 15. The loss of

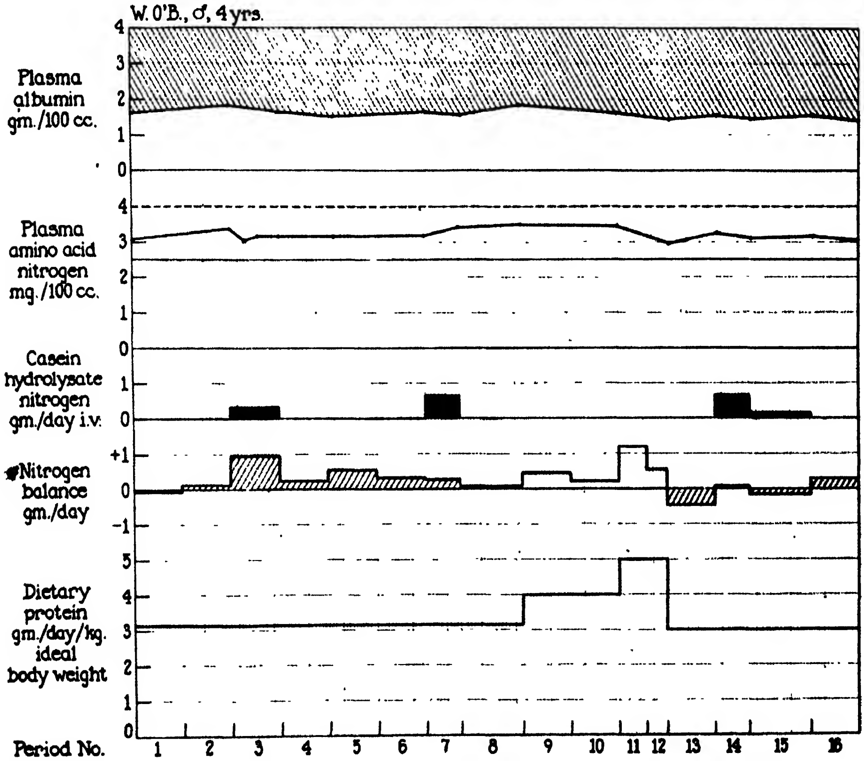

FIG. 6. Summary of nitrogen balance studies on W. O'B. Note the constant level of plasma albumin throughout the study although the nitrogen balance was positive throughout thirteen of sixteen periods. Normal average plasma amino acid nitrogen 4.50 mg. per 100 c.c.

nitrogen during period 13 followed a change in diet to a lower intake and does not represent a true lack of assimilation. The negative balance noted in periods 1 and 15, however, cannot be explained on the basis of dietary changes or infection, but must be a reflection of disturbances in nitrogen assimilation during these periods. The large positive balances for periods 9 and 11 again reflect a change in diet. A smaller amount of nitrogen was retained during these periods and also during periods 10 and 12 by W. O'B. than by R. Q. Again, in W. O'B. no marked effect upon nitrogen assimi-

lation was seen after amino acid administration. The nitrogen balance of E. S. was influenced during periods 9, 11, and 13 by changes in diet. The relatively large amounts of nitrogen retained during periods 9 and 11 were not lost during periods 10 and 12, and very rapid attainment of equilibrium after changes in the diet is indicated by the very small loss during period

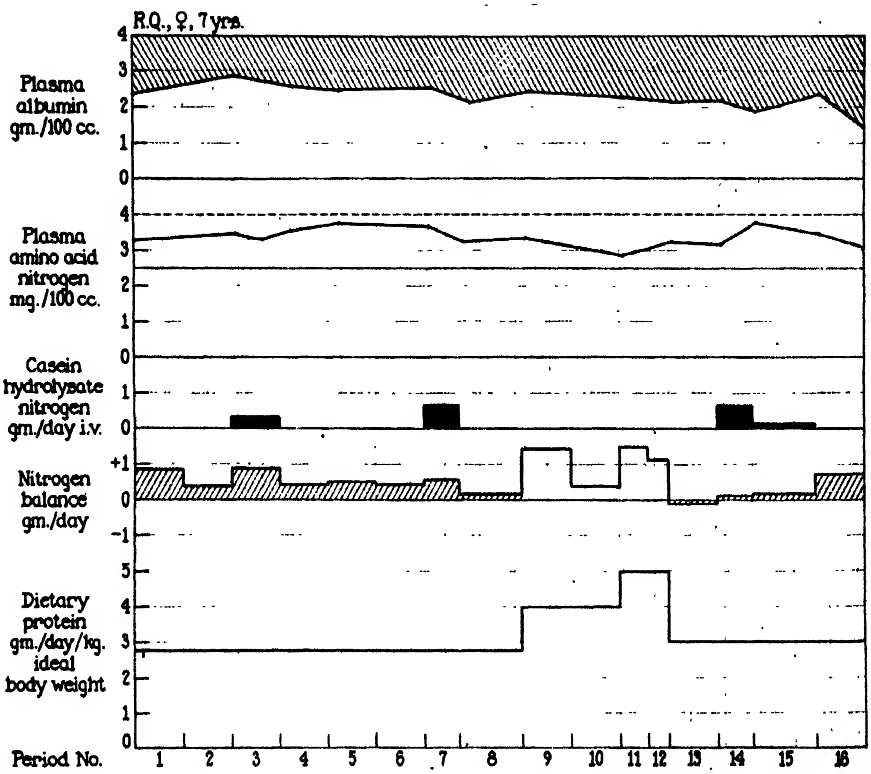

FIG. 7. Summary of nitrogen balance studies on R. Q. Note the gradual decrease in plasma albumin, although the nitrogen balance was positive. Retention of nitrogen during periods 9 and 11, following changes in protein intake, was indicated by the continuing positive nitrogen balance through periods 14 to 16. Normal average plasma amino acid nitrogen 4.50 mg. per c.c.

13. This patient showed a more marked response to amino acid administration than did any of the other patients studied. Her nitrogen assimilation increased most markedly during period 7, when the amino acids were given without the simultaneous administration of glucose. The negative nitrogen balance noted during periods 4 and 5 was not due to infection or diet change, but was due to the failure to assimilate nitrogen which is characteristic of nephrotic patients. J. C. was on a positive nitrogen bal-

ance, except during periods 6, 7, and 8. The negative nitrogen balance during period 6 preceded a nephrotic crisis which developed the first day of period 7 and another crisis complicated with infection during period 8. The development of a negative nitrogen balance as a characteristic precursor to nephrotic crises has already been described.[16] It is worth noting that there was no increased loss of nitrogen during period 8 as compared

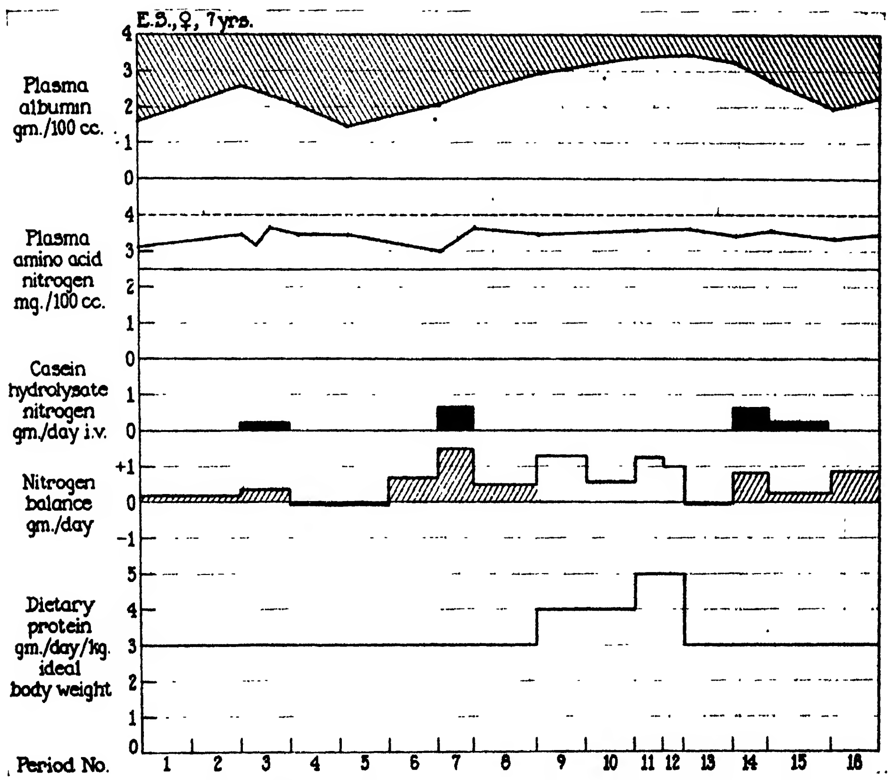

FIG. 8. Summary of nitrogen balance studies on E. S. Note the fall in plasma albumin level during periods 3, 14, and 15, although the patient was on a positive nitrogen balance. Normal average plasma amino acid nitrogen 4.50 mg. per 100 c.c.

with the preceding period, although the patient suffered from a severe infection during this time. The marked positive nitrogen balance after recovery from the crises and infection reflected an improvement in nitrogen assimilation during this interval. The nitrogen balance of this patient was not altered significantly by administration of amino acids.

No effects upon the level of the plasma proteins nor the degree of proteinuria were noted after amino acid injections. What was more surprising was the failure of the plasma albumin to increase in R. Q. after fifteen

weeks in positive nitrogen balance, and the marked improvement in plasma albumin in J. C. during period 8 when he was in negative balance. The fall in plasma albumin seen in E. S. while on a continuously positive nitrogen balance was not expected.

The average value of the urea clearance increased after administration of amino acids in three of the patients, E. S., J. M., and R. Q. (Figs. 1 to 3). There was no obvious effect upon the urea clearance of J. C. (Fig. 4). The increase in urea clearance was noted immediately after the injections were started. From three to five months elapsed following the final amino acid injection before the urea clearances in these patients returned to their pre-injection level. The increase in clearance was not due to a decrease in blood urea nitrogen during the injection periods, since this value did not change appreciably. There was no water diuresis following injection, although the average total urine output during the injection periods was somewhat higher than during rest periods. The same average urine volume increase has been obtained in these patients by administration of water alone. Under these circumstances, no change in the clearance level was observed.

The administration of amino acids did not change the proportion of urea plus ammonia nitrogen in the urine. The ratios remained remarkably constant in all four patients.

DISCUSSION

The marked retention of nitrogen was not expected on the diets which contained in éxcess of 3 Gm. of protein per kilogram of ideal body weight. In a previous study of nitrogen assimilation in five nephrotic children, both the relative and absolute assimilation of nitrogen decreased when more than 3 Gm. of protein per kilogram was fed.[2] Subsequent experience in this clinic with very high protein diets for these children has substantiated these observations. Maroney and Johnston[17] found decreased assimilation of nitrogen in normal children when more than 20 per cent of the total calories were furnished by protein. On the other hand, the normal child can readily maintain himself in positive nitrogen balance with only 2 Gm. of protein per kilogram, while the nephrotic child requires at least 2.5 Gm. of protein per kilogram. The inability of the nephrotic child to maintain himself in nitrogen equilibrium on a diet adequate for a normal child cannot be accounted for on the basis of nitrogen lost as protein in the urine. Increase of minimal nitrogen requirement in these patients simulates in many ways the increased requirements of nonnephrotic patients with fever or other intoxication. Since the behavior of the nephrotic child in regard

to nitrogen assimilation parallels that observed in toxic conditions, for convenience, such states, in which a negative nitrogen balance exists in a nephrotic patient with an adequate intake of dietary protein, may be characterized as those of "toxic" nitrogen metabolism, with the important reservation that *no assumption is made concerning the cause of the accelerated nitrogen excretion.*

Unpublished data obtained by us on nonnephrotic patients with pneumonia and bronchiectasis indicated that, under such well-recognized toxic conditions, hypoaminoacidemia was not uncommon. Thus, the hypoaminoacidemia and nitrogen wasting seen in nephrotic patients become different facets of "toxic" nitrogen metabolism. Although the administration of amino acids to these nephrotic patients did not decrease the minimal nitrogen requirements nor dramatically reverse a patient from the "toxic" to the nontoxic type of nitrogen metabolism, this would be expected since the level of plasma amino acids remained subnormal, and, hence, the nitrogen metabolism remained of the "toxic" type with increased maintenance requirements. The simultaneous administration of glucose did not enhance the effects of the amino acids.

On the other hand, the amino acid injections apparently increased the capacity to assimilate nitrogen to an extent sufficient to overcome the toxic effects of a high protein diet. At the end of three weeks' feeding of the high protein diets, clinical effects of toxicity were noted in increasing pallor and loss of appetite. During the first two weeks, however, the effects of amino acid injections given one full week prior to feeding the high protein diets remained dominant. This assumption of continuing effect for a period of time after injection is not unreasonable, as it was pointed out in a discussion of the effects upon the urea clearance that the clearance remained elevated for from three to five months after the injections were discontinued.

Following the high protein diets there was a sharp drop in the plasma albumin level of E. S., although she remained on a positive balance. Her rapid subsequent recovery when the optimal diet was restored and the fact that she made a complete recovery from her disease in the next two months indicate that the regulation of protein level in the blood of nephrotic patients may be one of the first mechanisms deranged in the disease. This partial independence of the plasma albumin level in relation to total nitrogen exchange was shown also by J. C., who exhibited a rise in plasma albumin during period 8 while on a negative nitrogen balance and a subsequent gradual fall during the last few periods while on a positive nitrogen balance. Since it is thus apparent that, over short intervals, the plasma albumin level may move in a direction counter to the general nitrogen

assimilation, failure to find improvement in plasma albumin levels immediately after injection sheds no light on the utilization of the injected amino acids. That they shared the fate of dietary protein was indicated by the constancy of the ratio of urea plus ammonia nitrogen to total non-protein nitrogen in the urine. Presumably, nitrogen retained was first used to restore depleted tissue proteins and to satisfy growth requirements, and only under unusual circumstances, such as prevailed in J. C. during and immediately after his hemolytic streptococcus infection, was the retained nitrogen diverted to the manufacture of plasma proteins. No deductions can be drawn from these studies as to the nutritional adequacy of the amino acid mixture used since no attempt was made to use this as the sole or major portion of nitrogen supplied.

The rise of the urea clearance following amino acid injection seemed to correspond to the rise previously observed in this type of patient when the diet was changed from a low to a high protein content.[18] The same changes cannot be induced by feeding urea. In a study on dogs, Van Slyke, Rhoads, Hiller, and Alving[19] found the change in urea clearance observed when the protein in the diet was increased to be due to an increased renal blood flow. It would appear that the stimulating factor under these circumstances may have been the increased supply of amino acids. Further studies on this point are at present under way in this clinic.

CONCLUSIONS

Four nephrotic children, two boys, aged 4 and 6 years, and two girls, each aged 7 years, have been observed on a nitrogen balance study for fifteen consecutive weeks.

Procedures used in dissolving and storing a suitable casein hydrolysate for intravenous use were described.

The intravenous administration of amino acids as a casein hydrolysate caused flushing of the skin and a sensation of warmth. When the infusions were given rapidly or large quantities administered, in addition to the above reactions, nausea and vomiting were common. No febrile reactions were encountered.

Nitrogen given as intravenous amino acids was assimilated and retained as well as, or better than, similar amounts of nitrogen fed as protein. After amino acid injections, the capacity of nephrotic patients to assimilate high protein diets was markedly increased over that previously observed. Increased effects of amino acids were not obtained by the simultaneous administration of glucose.

The injections had no effect upon the ratio of urea plus ammonia nitro-

gen to nonprotein nitrogen in the urine, plasma protein level, or fasting plasma amino acid level.

In three of the four patients the administration of amino acid intravenously produced an increase in the urea clearance which persisted for from three to five months after injections had been discontinued.

The nature of the inability of nephrotic patients to assimilate nitrogen was discussed.

REFERENCES

1. Farr, L. E., and MacFadyen, D. A.: Am. J. Dis. Child. **59:** April, 1940.
2. Farr, L. E.: Am. J. M. Sc. **195:** 70, 1938.
3. Van Slyke, D. D., and Meyer, G. M.: J. Biol. Chem. **16:** 197, 1913.
4. Elman, R.: Proc. Soc. Exper. Biol. & Med. **36:** 867, 1937.
5. Farr, L. E., and MacFadyen, D. A.: Proc. Soc. Exper. Biol. & Med. **42:** 444, 1939.
6. Hiller, A.: Proc. Soc. Exper. Biol. & Med. **24:** 385, 1927.
7. Van Slyke, D. D., and Kugel, V. H.: J. Biol. Chem. **102:** 489, 1933.
8. MacFadyen, D. A., and Van Slyke, D. D.: In preparation.
9. Van Slyke, D. D., Page, I. H., Hiller, A., and Kirk, E.: J. Clin. Investigation **14:** 901, 1935.
10. Van Slyke, D. D.: J. Biol. Chem. **81:** 235, 1927.
11. Howe, P. E.: J. Biol. Chem. **49:** 109, 1921.
12. Peters, J. P., and Van Slyke, D. D.: Hemoglobin by the Oxygen Capacity Method. Quantitative Clinical Chemistry, Vol. II, Methods, Baltimore, 1932, Williams and Wilkins, p. 337.
13. Addis, T., and Shevky, M. C.: Arch. Int. Med. **30:** 559, 1922.
14. Addis, T.: J. A. M. A. **85:** 163, 1925.
15. Cox, W. M., Jr., and Mueller, A. J.: Proc. Soc. Exper. Biol. & Med. **42:** 658, 1939.
16. Farr, L. E.: Am. J. Dis. Child. **58:** 939, 1939.
17. Maroney, J. W., and Johnston, J. A.: Am. J. Dis. Child. **54:** 29, 1937.
18. Farr, L. E.: J. Clin. Investigation **15:** 703, 1936.
19. Van Slyke, D. D., Rhoads, C. P., Hiller, A., and Alving, A.: Am. J. Physiol. **110:** 387, 1934.

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STUDIES ON "ESSENTIAL" HYPERTENSION

III. The Effect of Nephrectomy upon Hypertension Associated with Organic Renal Disease

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Arterial hypertension is found in patients whose kidneys exhibit a variety of lesions and who suffer from renal insufficiency. Similar lesions have been found in hypertensive patients who are free from renal insufficiency.[5, 12a, b] That these lesions play a part in the existence of hypertension is shown by the fall in blood pressure which has been reported to follow removal of a single affected kidney. Butler was the first to record this result in 2 children suffering from pyelonephritis.[4] Similar occurrences have been observed both in children and adults (Table 1). Of the 12 reported, 7 cases were in children. Only 3 were observed for more than a year and all were improved. One of the 5 adults was examined over a period of 11 years and remained free from hypertension for 10; finally, however, it recurred.* The other cases were published before an adequate interval had elapsed. The late results of nephrectomy in adults are therefore uncertain, although in every instance the immediate effect was a lowering of blood pressure for a matter of months. The inference that can be drawn from all the information now available constitutes, accordingly, an unsatisfactory basis for drawing a conclusion on the value of this operative procedure; for the most part the cases were in young persons; the duration of observation was short; the end results are uncertain. The effects of operation observed in the period of weeks or months afterwards make desirable a further analysis of cases so treated. To follow out this plan is reasonable because renal affections have been found to be so common in cases of arterial hypertension. Nephrectomy has therefore been performed in 7 instances.

* A review of the older urologic literature has failed to reveal the effect of nephrectomy upon the arterial pressure, or of cases similar to those which have recently been published. Barney and Suby,[2] however, mention a series of 305 cases of pyelonephritis and hydronephrosis in the records of the Massachusetts General Hospital, of which 25% exhibited hypertension. It is hoped that other similar statistics will soon be available in the literature.

Two patients were not improved and have subsequently died; 2 were improved for some time (16 and 11 months); 1 was temporarily improved; and in 2, no change has been observed. In no instance has hypertension disappeared. These 7 cases will be discussed separately.

Patients were selected in whom severe arterial hypertension was associated with unilateral renal disease accompanied by marked diminution of function of the affected kidney but not by renal insufficiency. In 4 there was evidence of disease of the opposite kidney of lesser degree, and in 3 no other lesion was demonstrated. It must be emphasized that little difficulty was encountered in finding such cases; they are common among younger individuals.

Case Reports. CASE 1.—(Hosp. No. 10,354). H. S., aged 35, was a taxi driver who had suffered from arterial hypertension for 2 years. His mother died of high blood pressure at the age of 67. In 1926 he experienced an attack of renal colic and hematuria from which he rapidly recovered. There were 2 similar attacks during the next 2 years. His blood pressure was normal in July, 1936, but in March, 1937, his systolic pressure was 190 mm. Hg.

In December, 1937, examination revealed the presence of a small hemorrhage at the temporal side of the left optic disk. The vessels of the retina were narrow and slightly tortuous with obvious irregular areas of constriction. The heart was enlarged in Roentgen ray photographs; there was diffuse thickening of the radial arteries. His systolic pressure was 210, his diastolic 130 mm. Hg. The urine showed a trace of albumin with many white cells, singly and in clumps. The clearance of urea* was 84% of normal. Shortly after this he suffered an attack of pain in his left costovertebral angle; urination was painful. A small amount of hydronephrosis existed on the right (pyelogram after intravenous injection†) and in the left renal pelvis was a large, rounded, apparently movable stone. In February, 1938, reëxamination of the ocular fundi showed a small fresh hemorrhage near the right optic disk and a scar on the temporal side of the left one. Cystoscopic examination was performed and separate specimens from the two kidneys showed that the clearance of urea on the right was 37.7% of normal; on the left, 22.6%. Maximal specific gravity of the urine‡ was 1.022; there were very many white blood cells, single and in clumps, and culture showed the presence of *Escherichia coli*. He was transferred to the Squier Urological Clinic for operation which was performed March 7th. The left kidney was found to be granular and smaller than normal, and its capsule was thick, fibrous and densely adherent. The stone was found fixed in the ureter, which was dilated above it, and there was an artery crossing the uretero-pelvic junction, producing angulation. The stone was removed and nephropexy done. Because of infec-

* By "clearance of urea" is meant that of Van Slyke.

† By "pyelograms after intravenous injection" is meant Roentgen ray photographs of the genito-urinary tract after the intravenous injection of Diodrast.

‡ By "maximal specific gravity of the urine" is meant the non-protein specific gravity after rigid restriction of fluids for 36 hours.

tion in the wound and the development of a huge hydronephrosis and hydro-ureter on the left side, nephrectomy was later performed. Following discharge he developed an attack of acute pulmonary edema, from which he rapidly recovered. In June, 1938, cultures of the urine again demonstrated the presence of *E. coli*, and no change in his blood pressure had occurred. A considerable amount of pus was found in his urine which did not disappear on treatment.

In August, 1938, he was readmitted because of several attacks of paroxysmal nocturnal dyspnea. Bilateral papilledema of two diopters with perivasculitis was seen in the ocular fundi, and there were many flame-shaped and linear hemorrhages with areas of exudate and scars. The urinary infection persisted in spite of therapy with sulfanilamide.

The symptoms of heart failure increased, his clearance of urea fell, and his blood urea nitrogen became elevated. He died on December 2, 1938, of cardiac and renal failure.

Description of Specimen. The left kidney measured 9 by 4 by 2.5 cm. The capsule was thickened, shaggy, and densely adherent to the parenchyma. The renal substance measured 1 cm. in thickness and was opaque and gray in appearance. There was marked dilatation of the pelvis and calyces. On microscopic section many areas of extensive fibrosis were seen through which there was infiltration with lymphocytes. Many glomeruli were fibrotic; in others Bowman's capsule was thickened and adherent to the glomerular loops. The tubules were dilated and atrophied and contained purulent casts and calcareous débris. There was marked thickening of the intima and media of the smaller arteries and arterioles, some being totally occluded.

Autopsy: Right Kidney. The kidney weighed 265 gm. The cortex appeared hypertrophied, and the calyces stood out distinctly and were slightly dilated, as was the lower ureter. On microscopic section, the tubules were seen to be markedly dilated, extending to the surfaces of the cortex in many instances. The glomeruli were decreased in number and appeared to be arranged in groups radially from the calyces instead of being scattered irregularly throughout the cortex as is found in the normal kidney. In these radial areas there was usually a great deal of fibrosis and between them were dilated tubules. The glomeruli themselves were thickened and Bowman's capsule was distended. There was no congestion in the kidney, but the vessels, particularly the arteries, showed a thickened medial wall.

Anatomic Diagnosis. Pulmonary infarction; marked hypertrophy of heart; chronic passive congestion of liver; diffuse arteriolar sclerosis; arteriolonephrosclerosis, mild; cyst of pars intermedia of pituitary gland.

Comment on Case 1. Arterial hypertension exhibiting a "malignant" course was associated with bilateral organic renal disease. There was a calculus in the left kidney, hydronephrosis, aberrant renal artery constricting the uretero-pelvic junction, and pyelonephritis. There was also right hydronephrosis of slight degree with infection. Removal of the left kidney resulted in no change in the rapid, downhill course. Death occurred from cardiac and renal failure. There was marked vascular disease in the kidney removed at operation, but it was minimal in the remaining kidney. In addition, a cyst of the pituitary was found.

TABLE 1

Summary of Cases Previously Reported in the Literature

Reported by	Age.	Sex.	Preoperative range of B. P.	Postoperative range of B. P.	No. of mos. followed.	Maximal urine concentration, specific gravity.	Phenolphthalein excretion 2 hrs. (%).	Renal lesions.	Result.	Remarks.
Butler[4]	7	♂	168/110 122/90	115/75 100/70	20	1.024	55	Pyelonephritis	Improved	Moderate arteriolar sclerosis of kidney
	10	♀	190/120	110/70 92/60	3	—	Normal	Pyelonephritis; dilated pelvis	Improved	Marked arteriolar sclerosis of kidney
Pincoffs and Bradley[11]	2	♀	180/110+ 150/110	130/100	3	1.032	80	Adenosarcoma	Slight improvement	Patient died of recurrence. B. P. rose to former level
	2	♂	140/100 120/80	88/60	7	1.028	80	Adenosarcoma	Temporary improvement	B. P. normal for 6 mos., but returned to previous level
Barker and Walters[1]	42	♂	200/140 170/120	144/100 128/90	2	—	Blood urea normal	Atrophic pyelonephritis	Improved	Thickened arcuate and interlobar arteries. Scarring of kidneys
Leadbetter and Burkland[8]	5½	♂	174/120 132/82	120/74 96/70	1(?)	1.026	90	Ectopic kidney; partial occlusion renal artery by plug of smooth muscle	Improved	No intrarenal vascular disease
Boyd and Lewis[3]	31	♂	225/140 165/100	150/100 125/75	6	1.017	70	Infarction of kidney	Improved	Narrowing of renal arteries
Quinby[*]	14	♂	250/170	130/?	12+	1.015	15	Hydronephrosis; chronic pyelonephritis	Temporary improvement	Marked destruction of kidney

Crabtree[8]	27	♀	210/? 180/?	190/? 120/76	134	—	45	Chronic pyelonephritis; stricture of ureter	Improved 10 yrs.	Return of hypertension 11th year
	40	♂	170/120 100/72	120/98 100/70	2	1.028	50	Calculi; pyelonephritis	Improved	Narrowing of intrarenal arteries
Barney and Suby[3]	10	♀	200/170 185/130	110/70 98/60	21	—	61	Pyelonephritis with atrophy	Improved	Marked renal vascular disease
McIntyre[*]	35	♂	180/104 168/94	150/94 128/78	9	1.024	35	Pyelonephritis; double kidney; hydronephrosis	Improved	Narrowing of main renal artery by arteriosclerosis

* Reported by Crabtree.[8]

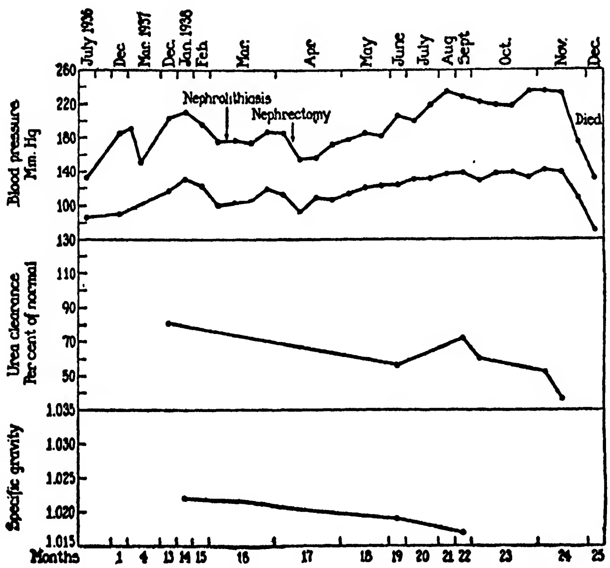

CHART 1. Showing course of Case 1. Upper line is average systolic; lower, average diastolic pressure. The urea clearance refers to that of Van Slyke. Specific gravity refers to the maximal specific gravity of the urine after fluids were restricted for 36 hours. Months refers to duration of arterial hypertension.

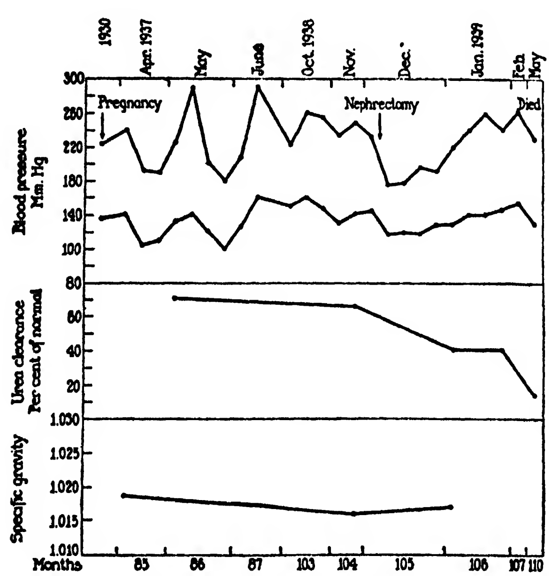

CHART 2. Showing course of Case 2. Notations same as Chart 1. Blood pressure was said to have been normal previous to point marked "pregnancy."

CASE 2.—(Hosp. No. 10,598). A similar event occurred in the case of B. W., a 30-year-old housewife, who had suffered from hypertension for 8 years. Her mother died of hypertension and her father's sister exhibits it. At the age of 8 she became ill for several months because of "pus in her kidneys." Pyuria, low grade fever, and loss of weight occurred. She continued to show pus in her urine on occasional examinations for the next 12 years, but was aware of no other symptoms. Her blood pressure was normal at the age of 21, when she was married. She was soon pregnant. In the sixth month her ankles became swollen, she suffered severe headaches and her blood pressure was found to be elevated. Nausea, vomiting, headache, edema, Pal's crises, hypertension and albuminuria made termination of the pregnancy necessary and a live infant was delivered. Her blood pressure did not return to normal. At age 29 her systolic pressure varied from 260 to 210, her diastolic from 150 to 120 mm. Hg. Her heart was only slightly enlarged in Roentgen ray photographs. One scar was seen in the retina and the arteries were tortuous. *E. coli* was found on culture of the urine.

Pyelograms after intravenous injection disclosed ptosis of the right kidney with slight dilatation of the renal pelvis. The shadow of the left kidney was small. Cystoscopic examination with retrograde pyelography showed a markedly contracted left kidney with a dilated renal pelvis and upper ureter. Studies of the functions of the two kidneys were as follows:

	Right.	Left.
Phenol red excretion (20 min.)	5.8%	0
Clearance of urea	24%	4%
Albumin	0	+

She was transferred to the Squier Urological Clinic where cultures of the urine from the *right* ureter showed the presence of Gram-negative bacillus of the colon group, while that from the left was sterile. Her systolic pressure varied from 270 to 230 mm. and her diastolic from 170 to 132. The left kidney was removed Dec. 2, 1938. It was very small (12 gm.) and was markedly adherent to the surrounding fatty tissue. The pelvis was dilated, thin, and friable, the ureter small.

Immediately following operation her blood pressure fell temporarily to a level between 180 and 140 mm. systolic and 120 and 110 diastolic, but 3 weeks later it was 240 to 200 systolic and 160 to 110 diastolic, and 2 months after operation it remained at the pre-operative level. The clearance of urea had fallen to 41% and exudate, hyperemia of the optic disks, and slight papilledema were noted in the ocular fundi. Cultures of the urine again demonstrated the presence of *E. coli*. She died May 15 of renal failure.

Description of Specimen. The kidney weighed 12 gm. The pelvis was dilated and very thin. There were many dense, radial scars of fibrous tissue extending from the medulla to the capsule, and the cortex was thin. Microscopic section showed dense fibrosis of the parenchyma. There was marked thickening of the intima and media of all the vessels, and in many instances their lumina were obliterated. In a few of the tubules purulent material was seen.

Autopsy: Right Kidney. The kidney weighed 200 gm. On section, no cortex was evident, and calyces in five places extended directly to the capsule. The renal parenchyma appeared to be made up entirely of medulla interspersed with fibrous tissue. The calyces, pelvis, and ureter were dilated. On microscopic section, the normal architecture of the kidney was drastically altered. The glomeruli were reduced in number, many

being atrophic and some calcified. The normal line of demarcation between medulla and cortex was destroyed and many groups of glomeruli were definitely in the medullary area. There was extensive irregular dilatation of the tubules with many areas of degeneration, some being infiltrated with numerous round cells and fibroblasts. Almost all of the arteries and arterioles showed marked thickening and sclerosis of the media.

Comment on Case 2. Infection of the kidney in childhood apparently became chronic. Pregnancy resulted in toxemia with hypertension. The left kidney was found to be contracted, and function impaired. Removal

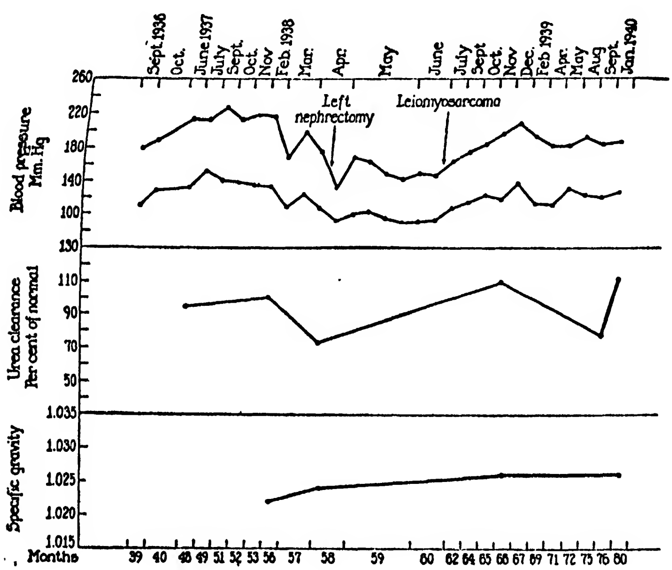

CHART 3. Showing course of Case 3. Notations same as in Chart 1. At the point marked "leiomyosarcoma" a tumor was removed from the stomach. For fuller explanation see case report.

of this kidney failed to change the course of hypertension, and death occurred from renal failure 5 months later. There was advanced arteriolar sclerosis of both kidneys. Pyelonephritis associated with hydro-ureter and slight hydronephrosis were present in the functioning kidney.

CASE 3.—(Hosp. No. 10,237). A. L., aged 30, was a stenographer. Her father suffered from hypertension and his brother died as a consequence of it. At the age of 20 she experienced a number of attacks of pain in her left lumbar region associated with nausea, vomiting, and hematuria. A moderate degree of hydronephrosis was found on the left, with angulation of the ureter at the uretero-pelvic junction and some diminution of function. Her blood pressure was first found slightly elevated at age 24; at 26 was definitely so.

In June, 1937, she complained of severe headaches, moderate dyspnea on exertion, and palpitation. Her heart was slightly enlarged in Roentgen ray photographs. The

clearance of urea was 97% of normal. In September, a scar was seen in the ocular fundus and there was evidence of perivasculitis. In February, 1938, pyelograms after intravenous injection showed moderate hydronephrosis on the right with ptosis of the kidney and angulation of the ureter at the uretero-pelvic junction; the outline of the left renal pelvis was not seen. Cystoscopic examination with retrograde pyelography demonstrated a large hydronephrotic kidney on the left, with sharp angulation at the uretero-pelvic junction. The average clearance of urea on the right was 89% of normal; on the left, 21%. Albumin was present in the urine from the right but not in that from the left kidney. She was transferred to the Squier Urological Clinic where left nephrectomy was performed March 14. The kidney was enlarged and the cortex was thin. There were several anomalous vessels about the hilum, and the ureter was kinked about one of these which entered the lower pole. Her blood pressure the day of operation was 270 systolic and 150 diastolic, but on the following day it had fallen to 130 systolic and 90 diastolic, and during her convalescence varied from 120 to 176 systolic and from 80 to 108 mm. Hg. diastolic. In May, 1938, she became anemic and the presence of blood was discovered in the stools. Roentgen ray photographs of the gastro-intestinal tract revealed a large, round defect on the lesser curvature of the stomach. Exploratory laparotomy was performed June 13 at the New York Hospital by Dr. Ralph Bowers, and a portion of the stomach containing a mass was resected. The tumor was a leiomyosarcoma, but there appeared to be no involvement of adjacent lymph nodes. Convalescence from this operation was uneventful. Her blood pressure before operation was 160 systolic and 100 diastolic, but immediately afterwards and during her postoperative course rose to 180 systolic and 110 diastolic, and 6 weeks later was 162 systolic and 106 mm. Hg. diastolic. In November, 1938, her systolic pressure varied from 200 to 184 and her diastolic from 120 to 110. Since then her blood pressure has remained at this level. There have been no symptoms suggesting recurrence of the neoplasm of the stomach, and she has noticed a complete disappearance of her headaches.

Anatomic Diagnosis of Kidney. Hydronephrosis; chronic pyonephrosis; fibrosis and atrophy of kidney; arteriolar sclerosis, moderately severe.

Comment on Case 3. Arterial hypertension was associated with bilateral hydronephrosis and right nephroptosis. The left kidney was markedly damaged. Nephrectomy resulted in a sustained fall in blood pressure, which did not reach normal levels and which has approached the preoperative range. The removal of a sarcoma of the stomach did not lower the level of the blood pressure. There was symptomatic improvement following nephrectomy. A moderately severe grade of vascular disease was found in the affected kidney.

CASE 4.—(Hosp. No. 8947). M. B., was a nurse, aged 33. A maternal aunt died of apoplexy following hypertension. Her general health was excellent until the age of 23 when she began to complain of pain in her right flank and groin which at times became severe. Cystoscopic examination was made. Marked ptosis of the right kidney with slight hydronephrosis was found in Roentgen ray photographs. Following this procedure the attacks recurred, coming on as often as twice a month, relieved by cystoscopy with catheterization of the renal pelvis. The frequency of the attacks gradually became less over the next few years, but during the past 2 became greater.

Her blood pressure was first found elevated at the age of 24 and a few months later headaches appeared. At the age of 25 her systolic blood pressure was 180 mm. Hg.; at 27 it was 240, and at 28 her systolic was 198, her diastolic 136 mm. Hg.

In 1934, when she was admitted to this hospital, the physical examination was not remarkable. There was moderate obesity. The ocular fundi were normal. The heart was slightly enlarged in Roentgen ray photographs. The peripheral vessels were not thickened. The clearance of urea was 119% of normal and the maximal specific gravity of the urine was 1.036. There were no albuminuria or abnormal microscopic elements except for a few hyaline casts Her basal metabolic rate was −25% of normal. After 2 weeks' rest in bed her blood pressure fell from a level of 200 mm. Hg. systolic, and 135 mm. Hg. diastolic, to 160 and 95, but rose again when she became active.

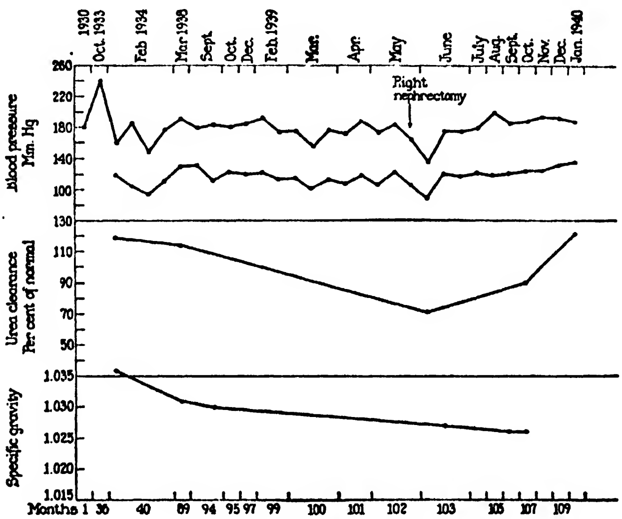

CHART 4. Showing course of Case 4. Notations same as Chart 1

Four years later, when she was 32, her systolic blood pressure remained at a level of 190; her diastolic at 130 mm. Hg. The heart was slightly larger in Roentgen ray photographs. The ocular fundi were normal. The clearance of urea was 115%, and the maximal specific gravity of the urine was 1.031. There was a small amount of albuminuria and many white blood cells in the urine. The basal metabolic rate was −20%.

Pyelograms after intravenous injection revealed an apparently normal left renal pelvis and ureter. On the right, the kidney was markedly ptotic, the pelvis being small and contracted, and situated below the iliac crest. Cystoscopic examination was therefore performed in October, 1938, and again in February, 1939, and the function of each kidney ascertained as follows:

	1938.		1939.	
	Right.	Left.	Right.	Left.
Phenol red excretion (10 min., 1938; 75 min., 1939)	2.9%	12.1%	16.1%	34.8%
Clearance of urea	21.0%	88.0%	28.0%	53.0%

Because of repeated attacks of pain in the right flank, nephrectomy on the right side was performed at the Squier Urological Clinic, June 10, 1939. The kidney was low in position, the ureter short, and the vessels entering the hilus elongated. The kidney itself was smaller than normal. Her blood pressure fell after operation to 138 systolic, 90 diastolic, but one month later it had risen to its preoperative level, where it has remained.

Description of Specimen. The kidney was small and atrophic, and a large artery and vein ran in a tortuous oblique course across the upper anterior portion. There were also two aberrant renal arteries, one at the upper and one at the lower pole. The pelvis was distorted, but the cortex was of normal thickness, except where several fibrous bands

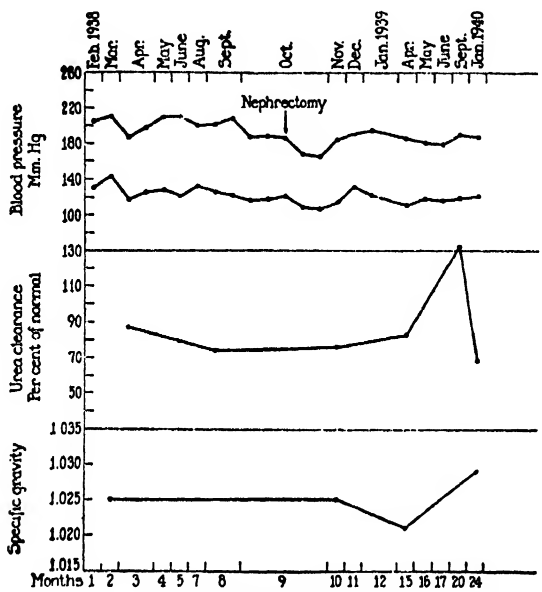

Chart 5. Showing course of Case 5. Notations same as Chart 1

extended to its surface. On microscopic section, Bowman's capsule was moderately thickened, and there was marked narrowing of the lumina of the smaller arteries and arterioles owing to sclerotic changes. Considerable fibrosis was evident.

Comment on Case 4. Arterial hypertension was associated with a unilateral renal affection which probably preceded the elevation of blood pressure. Although total renal function was normal, it was diminished in the affected kidney which was contracted and ptotic. Removal of this kidney 9 years after the onset of hypertension resulted in no change in blood pressure. Disease of the renal arteries was marked.

Case 5.—(Hosp. No. 10,399). The case of F. C., a 26-year-old housewife, was similar. Her mother suffered from arterial hypertension. During childhood she developed chronic urinary infection said to have been pyelitis. At the age of 25 she

experienced considerable emotional stress and began to notice marked nervousness. Her blood pressure was then found to be elevated, being 192 systolic and 130 mm. Hg. diastolic. Her heart was not enlarged. The ocular fundi were of normal appearance. Maximal specific gravity of the urine was 1.025, with slight albuminuria and cylindruria. Clearance of urea was 87% of normal. Retrograde pyelograms showed a small renal pelvis on the right, the calyces of which were club-shaped and distorted. Studies of the separate functions of the two kidneys were as follows:

	February, 1938.		September, 1938.	
	Right.	Left.	Right.	Left.
Phenol red excretion (10 min., Feb.; 30 min., Sept.)	0.4%	3.8%	1.3%	14.3%
Clearance of urea	17.0%	34.0%	3.0%	36.0%

Her systolic blood pressure then varied from 212 to 192, and her diastolic from 142 to 122 mm. Hg.

The right kidney was removed on October 18. It was small (25 gm.), hypoplastic, scarred, and densely bound down by perirenal and peripelvic adhesions. There was a large aberrant artery entering the upper pole. Immediately following operation her blood pressure fell to almost normal levels, but one month later her systolic pressure, during rest in bed, varied from 200 to 170, her diastolic from 124 to 98 mm. Hg. There was no change in renal function. During the next 16 months her systolic pressure varied from 196 to 180, and her diastolic from 136 to 114 mm. Hg. She has complained of no symptoms.

Anatomic Diagnosis of Kidney. Arteriolar sclerosis, moderate to severe; fibrosis of kidney; (?) chronic pyelonephritis, healed.

Comment on Case 5. Arterial hypertension was associated with a unilateral contracted kidney, which may have been the seat of chronic pyelonephritis sustained in childhood. Removal of this kidney resulted in a temporary fall in blood pressure. There was moderately advanced vascular disease in the affected kidney. The course of the disease does not appear to have been influenced.

CASE 6.—(Hosp. No. 10,523). G. R., a 33-year-old electrician, was known to have suffered from arterial hypertension for 3 years. His past history was uneventful. At the age of 28 his systolic pressure was 134 mm. Hg. and his diastolic 84; at 29, 138 and 90; at 30, 145 and 112; at 31, 138 and 110. In April, 1938, at age 32, it varied from 170 to 182 systolic and 130 to 144 mm. Hg. diastolic, and he was therefore, referred to this hospital (R. I. H.) complaining of daily headaches and excessive fatigue.

On examination, there was little of importance to be made out. His ocular fundi were not remarkable. His systolic blood pressure was 192 mm. and his diastolic 138. He was admitted August 14, 1938. The vessels of the retina were then seen to be hazy and indistinct along their margins. Maximal specific gravity of the urine was 1.025, and there was moderate albuminuria and cylindruria. Pyelograms after intravenous injection demonstrated ptosis of the right kidney with slight hydronephrosis and angulation of the ureter; none of the radio-opaque medium was excreted by the left kidney.

Cystoscopic examination was therefore performed on October 11, and retrograde pyelograms showed a huge hydro-ureter and hydronephrosis on the left, with constriction at the uretero-vesical junction. Studies of the functions of the two kidneys were as follows:

	Right.	Left.
Phenol red excretion (36 min.)	9.3%	0
Clearance of urea	50.0%	13%
Albumin	0	++

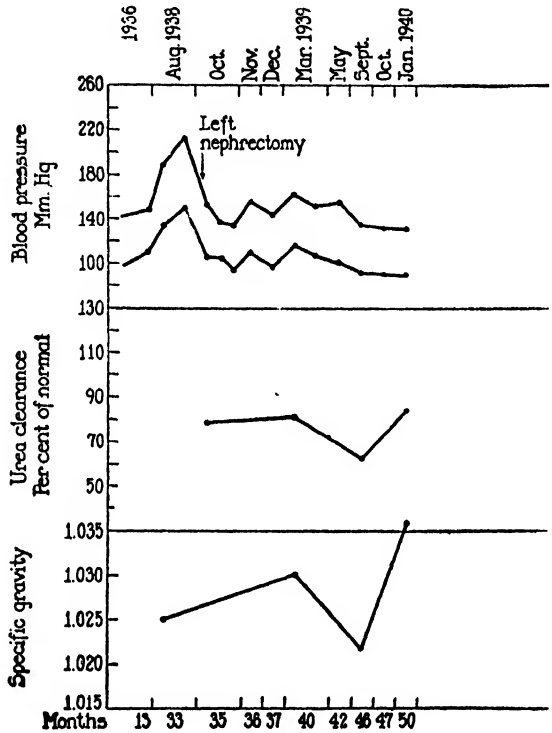

CHART 6. Showing course of Case 6. Notations same as Chart 1

In the ocular fundi at this time were slight papilledema and definite perivasculitis without exudate or hemorrhage. The clearance of urea was 82% of normal. A reaction to cystoscopy ensued, with fever, pain, and colic on the left side, which he stated was similar to an attack suffered at the age of 14. He was therefore transferred to the Squier Urological Clinic where left nephrectomy was performed on October 24, 1938. The kidney was densely adherent to the surrounding structures and the large and small intestines were bound down to the region of the renal pelvis. There was marked multiple angulation of the upper third of the ureter, which was dilated, thickened, and covered with fibrous bands. His blood pressure fell immediately to normal levels and his convalescence was uneventful. During the next 16 months his systolic pressure varied between 150 and 130, and his diastolic between 100 and 90 mm. Hg. He has noticed complete disappearance of headaches, has gained 9 kg. in weight, and no longer complains of fatigue.

Description of Specimen. The kidney weighed 60 gm. It was almost completely fibrotic. Very little renal tissue could be made out on gross inspection. The calyces were dilated and rounded, and the fibrous parenchyma was thickened. On microscopic section, marked fibrosis was seen, with perivascular lymphocytic infiltration. In certain areas glomeruli appeared normal, but many were congested and in the region of the scars none could be seen. There were purulent casts in the few tubules visible. The vessels were only slightly thickened as regards their intima and media.

Anatomic Diagnosis. Chronic pyelitis; hydronephrosis and hydro-ureter; fibrosis and atrophy; focal suppurative pyelonephritis; arteriolar sclerosis, minimal.

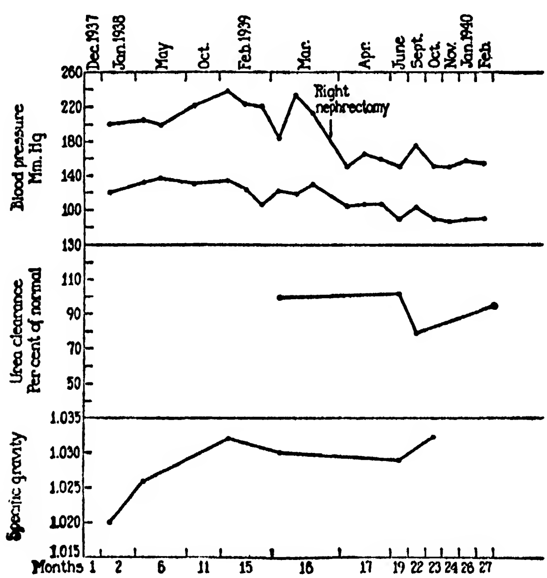

CHART 7. Showing course of Case 7. Notations same as Chart 1

Comment on Case 6. Arterial hypertension was associated with advanced left hydronephrosis and hydro-ureter, with constriction at the uretero-vesical junction. There were also right nephroptosis, ureteral angulation, and slight hydronephrosis. Removal of the severely damaged kidney has resulted in a marked and sustained fall in blood pressure for a period of 16 months. The course of the disease has been unquestionably altered. Only minimal vascular disease was present in the kidney.

CASE 7.—(Hosp. No. 10,475). A similar event was seen in M. A. a 20-year-old stenographer. At age 19, because of the complaint of increasing nervousness and palpitation following the death of her father, she was examined and her blood pressure was found elevated. She complained only of occasional headaches.

In May, 1938, the ocular fundi showed haziness of the nasal margins of both optic disks. The heart was slightly enlarged. Renal function was normal. Her systolic pressure varied from 220 to 194, her diastolic from 140 to 130 mm. Hg. Cystoscopic

examination was performed; the left kidney was able to excrete 10.5% of the injected phenol red in 20 minutes, the right, only 0.8%. Retrograde pyelograms showed that the upper calyx of the right renal pelvis was blunted and club-shaped, and only two calyces were present.

Cystoscopic examination was repeated in February, 1939, and the functions of each kidney measured separately:

	Right.	Left.
Phenol red excretion (30 min.)	0.5%	5.8%
Clearance of urea	21.0%	54.0%

The total clearance of urea was 100% of normal, and maximal specific gravity of the urine was 1.032. Her blood pressure was at a slightly higher level.

The right kidney was removed on March 24, 1939. It was found to be adherent to the surrounding tissues about its upper pole, which was scarred. Three months after operation her systolic pressure varied from 172 to 142 and her diastolic from 96 to 80 mm. Hg, at which levels they have remained. Renal function has remained normal. The most marked change to be noticed was the diminution of emotional instability. Instead of being a tense, excited, hysterical, uncoöperative individual, she had become quiet, apparently well-balanced, and moderately relaxed. This change of personality was noted immediately after operation and has persisted.

Description of Specimen. The kidney weighed 70 gm. Its surface was smooth, but it was small and about the upper pole were two depressed scars. On microscopic section only a minimal amount of vascular disease was seen. There was some fibrosis of the parenchyma, but glomeruli and tubules were of a normal appearance.

Anatomic Diagnosis. Hypoplasia of kidney; scarring of upper pole; arteriolar sclerosis, early.

Comment on Case 7. Arterial hypertension in a nervous young woman accompanied an undiagnosed condition of the right kidney. There was poor function of this kidney as compared with the left, and the renal pelvis was distorted. Removal of this kidney resulted in a lower level of blood pressure for 11 months.

Discussion. It is clear from these cases that removal of a diseased kidney does not consistently alter the course of arterial hypertension in a favorable direction. Only 2 cases were strikingly improved. These results indicate: 1, that the injured kidney was not the sole source of hypertension; and, 2, that other factors may occasionally be less important than the renal one. In every instance, however, removal of a kidney was followed by lowering of the level of the blood pressure for periods of weeks or months. It is difficult to believe that any major surgical procedure would have produced this result, since in some cases the improvement was prolonged, and in one instance (Case 3) operation of a much more extensive nature (partial gastrectomy) was followed by a rise in blood pressure. Some change in the pathologic physiology of the circulation occurs after

TABLE 2

Summary of Cases

Case No.	Name.	Sex.	Age at onset and duration previous to operation* (yrs.).		Ocular fundi.†	Preoperative range of B. P.‡	Postoperative range of B. P.	Last recorded B. P.	No. of mos. followed.§	Renal lesions.	Degree of renal vascular disease.	Remarks.
1	H. S.	♂	33	1	Hem.++ Scar+ A-S+	210/130 180/120	244/140 200/130	138/80	8	Left hydronephrosis, pyelonephritis and calculus. Rt. hydronephrosis and infection	++++(L) +(R)	No improvement. Death from cardiac and renal failure
2	B. W.	♀	21	8	Scar+ A-S+++	260/150 210/120	240/140 170/120	240/140	5	Left pyelonephritis	++++(L) +++(R)	No improvement. Death from renal failure
3	A. L.	♀	24	5	Scar+ A-S+ P-V+	230/150 196/126	200/120 120/80	182/110	23	Marked left hydronephrosis. Moderate right hydronephrosis, with ptosis	+++	Somewhat improved. B. P. considerably lower for 6 months
4	M. B.	♀	22	11	A-S++	190/135 170/100	190/125 180/120	192/124	8	Right ptosis and contracted kidney	+++	No improvement
5	F. C.	♀	<25>	1	Normal	212/142 192/122	200/124 140/90	182/122	16	? right pyelonephritis	+++	Sl. improvement in level of B. P.

6	G. R.	♂	30	2½	Pap.+ P-V+	212/150 190/138	150/100 120/80	132/90	16	Marked left hydronephrosis and hydro-ureter. Slight rt. hydronephrosis and ptosis	+	Considerable improvement
7	M. A.	♀	19	1½	Normal	240/150 194/120	150/100 120/90	150/100	11	? right pyelonephritis	+	Improvement to present time

* i.e., of hypertension.

† Hem. = hemorrhage; Scar. = scarring; A-S = arteriosclerosis; P-V = perivasculitis; Pap. = papilledema.

‡ Figures indicate average levels of blood pressure during a period of several days; single high or low readings are not noted. Upper figure is highest level; lower figure, lowest level maintained under observation.

§ Since operation.

nephrectomy, which we believe is due to removal of one factor at least causing hypertension.

All kidneys removed from these patients showed, in addition to a variety of lesions, inflammatory and non-inflammatory, varying degrees of arterial and arteriolar sclerosis. An attempt to grade the degree of vascular disease (Table 2) indicates that the least amount was found in those cases in which the best results were noted (Cases 6 and 7). It is not certain, however, that the degree of vascular disease in one kidney is the same as that in the other, there being a marked difference in Case 1. With the exception of Case 3, in which there was prolonged but temporary improvement, favorable results appeared proportional to the amount of disease of the renal arteries.

Further evidence that renal lesions are not the sole cause of arterial hypertension is their presence when the arterial pressure is not elevated. Some other factor beside these lesions contributes to the genesis of hypertension. In addition, continuance of the elevated blood pressure after removal of the affected kidney may be explained by the part vascular or other disease in the remaining kidney plays. The 2 patients who have died exhibited pyelonephritis as well as vascular lesions. Recent investigation in rats has shown that vascular disease in the unaffected kidney follows partial constriction of one renal artery,[13] and Goldblatt[7] has demonstrated its presence in other organs after bilateral arterial constriction. The pathogenesis of arteriolar sclerosis in the kidneys of human beings remains, however, unsettled. The occurrence of this lesion in almost all cases of "essential" hypertension[10] is nevertheless well recognized. It is still uncertain whether arteriolar disease is secondary to the renal lesion in cases similar to these, although animal experiments suggest such a relation.

From this study it is obvious that removal of a diseased kidney is not to be regarded as a procedure from which relief of hypertension can be expected to occur in all or in many cases. If arteriolar disease is secondary to the renal lesion or to hypertension and serves to maintain an elevated blood pressure which was initiated in the first place by a renal lesion, it would appear possible sometimes to obtain improvement by nephrectomy. A successful result might be expected if the lesion were confined to one kidney, and if arteriolar sclerosis of the other kidney had not progressed to an irreversible degree.

We suggest therefore the following criteria for selection of cases suitable for this form of therapy:

1. The onset of arterial hypertension should be known to have occurred recently. We place the time arbitrarily at 2 years.

2. The renal lesion should be confined to one kidney and should be of such a nature that diminution of function has occurred in that kidney.

3. Renal functions, as measured by the ability of both kidneys to concentrate urine and by the test of the clearance of urea, should be within normal limits.

4. Retinitis should be absent, and changes in the caliber of the vessels of the retina minimal.

5. Arterial pressure should be persistently elevated.

These criteria were followed only in Case 7, the others having exhibited either bilateral disease, hypertension of long duration, or some diminution of renal function. Unless suitable cases are selected, many kidneys may needlessly be removed (as in Cases 1, 2, and 4) without benefit. This experience has served to suggest that patients in whom long standing hypertension is present, or in whom a so-called "malignant" course has commenced are not good subjects for this form of therapy.

Summary and Conclusions. Seven patients exhibiting arterial hypertension associated with organic renal disease have been subjected to nephrectomy. Two were markedly improved, and 2 slightly improved, but all remain actually or potentially hypertensive.

This form of therapy may prove of benefit, but, it seems, only in patients in whom the existence of hypertension is of short duration and in whom arteriolar sclerosis of the other kidney is not advanced. Its use is limited, therefore, to a small number of individuals.

REFERENCES

(*1.*) *Barker, N. W.*, and *Walters, W.:* Proc. Staff Meet., Mayo Clin., **13,** 118, 1938. (*2.*) *Barney, J. D.*, and *Suby, H. I.:* New England J. Med., **220,** 744, 1939. (*3.*) *Boyd, C. H.*, and *Lewis, L. G.:* J. Urol., **39,** 627, 1938. (*4.*) *Butler, A. M.:* J. Clin. Invest., **16,** 889, 1937. (*5.*) *Cohn, A. E., Schroeder, H. A.*, and *Steele, J. M.:* Trans. Assn. Am. Phys., **54,** 82, 1939. (*6.*) *Crabtree, E. G.:* Trans. Am. Assn. Genito-Urin. Surg., **31,** 299, 1938. (*7.*) *Goldblatt, H.:* Harvey Lect., **33,** 237, 1937-38. (*8.*) *Leadbetter, W. F.*, and *Burkland, C. E.:* J. Urol., **39,** 611, 1938. (*9.*) *McIntyre, D. W.:* Ibid., **41,** 900, 1939. (*10.*) *Moritz, A. R.*, and *Oldt, M. R.:* Am. J. Path., **13,** 679, 1937. (*11.*) *Pincoffs, M. C.*, and *Bradley, J. E.:* Trans. Assn. Am. Phys., **52,** 320, 1937. (*12.*) *Schroeder, H. A.*, and *Steele, J. M.:* (*a*) Proc. Soc. Exp. Biol. and Med., **39,** 107, 1938; (*b*) Studies on "Essential" Hypertension. II. The Relation of Hypertension to Organic Renal Disease (to be published). (*13.*) *Wilson, C.*, and *Byrom, F. B.:* Lancet, **1,** 136, 1939.

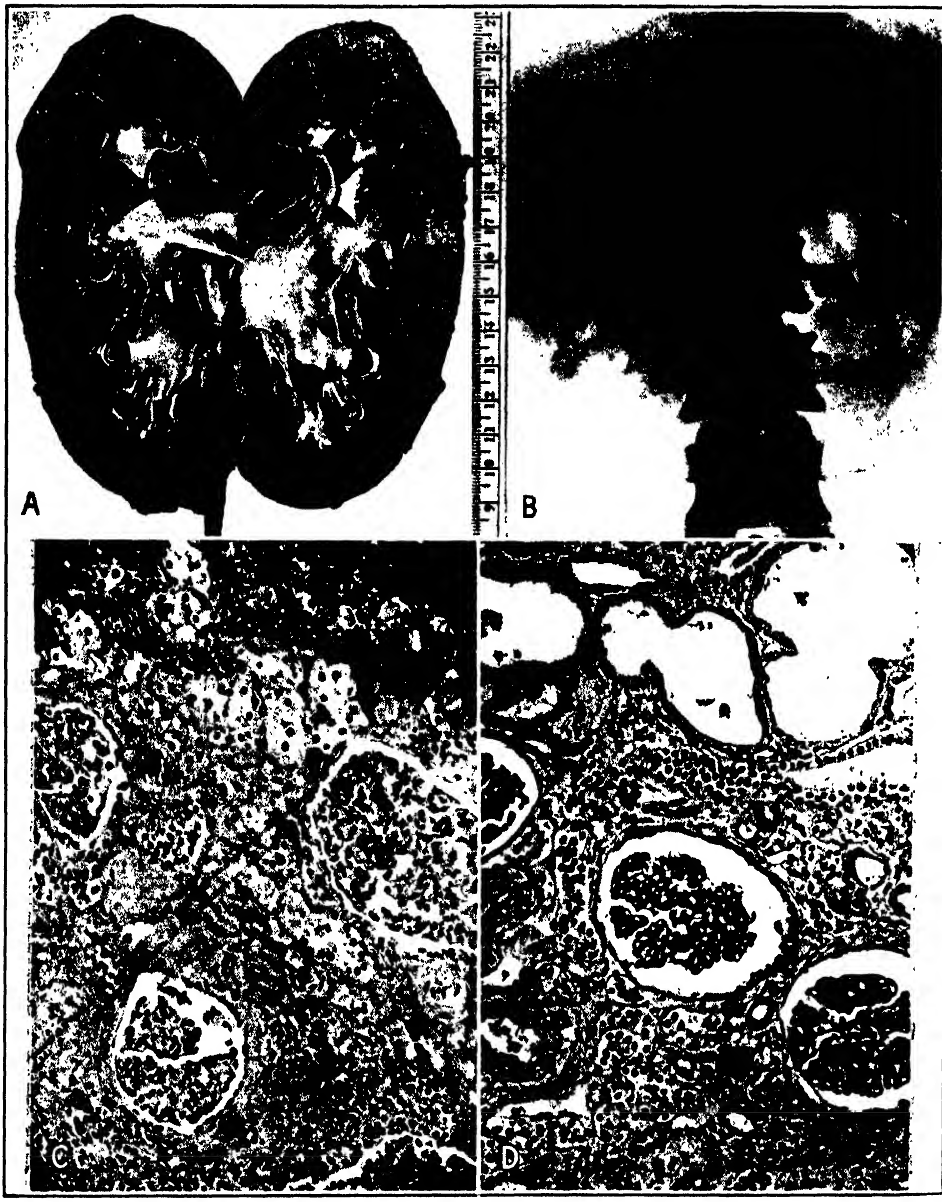

FIG. 1. Case 1. *A*, Right kidney removed at autopsy, showing almost complete obliteration of cortex; *B*, pyelogram after intravenous injection, showing calculus in left renal pelvis, and slight degree of hydronephrosis on right; *C*, microphotograph of left kidney. Note advanced vascular disease, tubular atrophy, and infiltration of parenchyma with lymphocytes. *D*, Microphotograph of right kidney. Some of the tubules are dilated, the glomeruli are shrunken, and there is very little disease of arteries and arterioles.

FIG. 2. Case 4. A, Pyelogram after intravenous injection, showing ptosis and distortion of right renal pelvis; B, microphotograph of a section from the right kidney. Vascular disease is moderate and there is some fibrosis of the parenchyma.

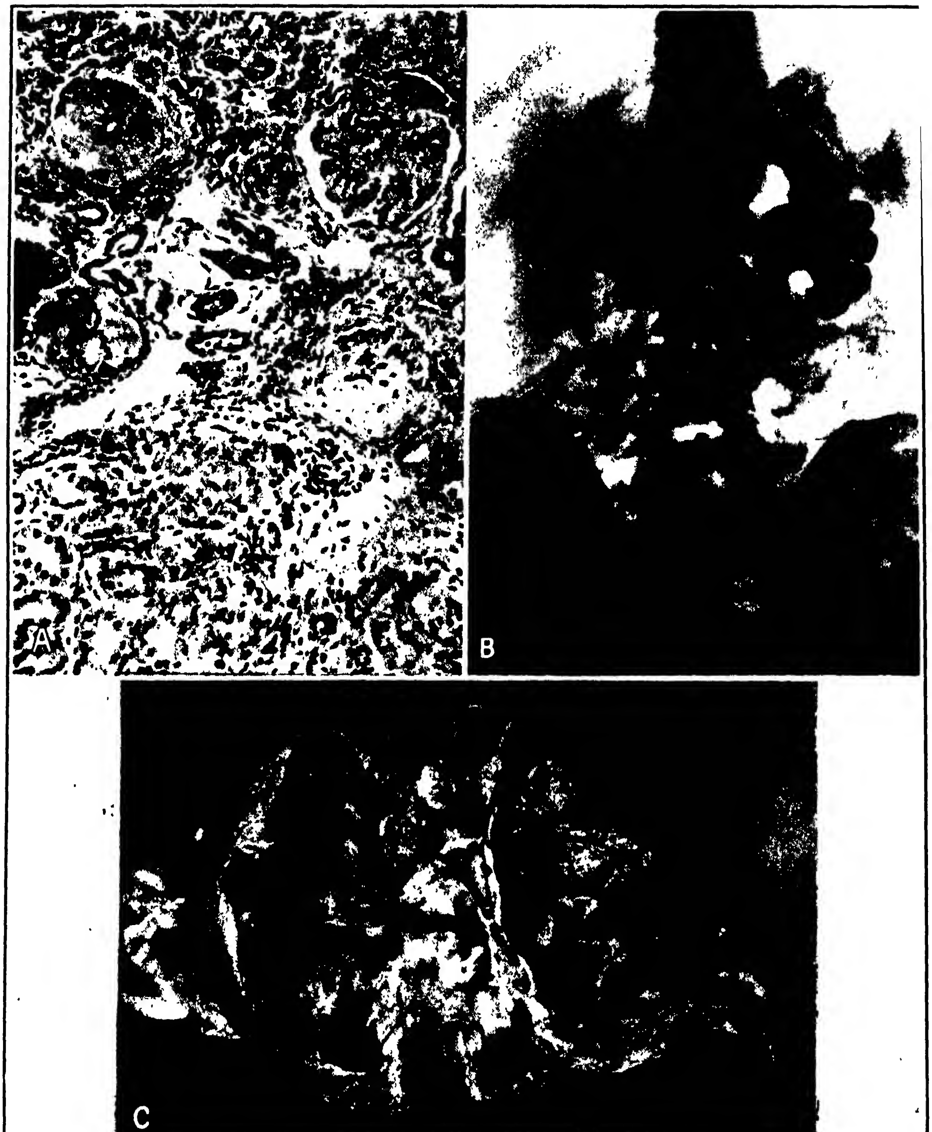

FIG. 3. Case 6. *A*, Microphotograph of left kidney. There is fibrosis, atrophy of tubules and glomeruli, and lymphocytic infiltration, with minimal involvement of arterioles. *B*, Retrograde pyelogram of left kidney, showing hydronephrosis and hydro-ureter. *C*, Photograph of this kidney, showing almost total destruction of parenchyma. Section has been removed from the upper border.

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INCREASED UROBILINOGEN EXCRETION AND ACUTE HEMOLYTIC ANEMIA IN PATIENTS TREATED WITH SULFAPYRIDINE[1]

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The occurrence of acute hemolytic anemia in patients receiving certain sulfonamide compounds (1) has been the subject of numerous reports. The mechanism responsible for the development of the anemia is unknown. However, it has been shown by Brownlee (2), Rimington (3), and Rimington and Hemmings (4) that many of the sulfonamide drugs derange the metabolism of pigments associated with blood formation and destruction. Rimington has emphasized the fact that many of these compounds are capable of being oxidized to hydroxylamine and suggests that this oxidation product may be responsible for the breakdown of red blood cells which occasionally follows the administration of the sulfonamide group of chemicals.

During the course of administration of sulfapyridine to patients with pneumonia, acute hemolytic anemia was observed and, consequently, a study of the incidence of hemolysis following administration of the drug was undertaken.

It has been shown by a number of investigators that the estimation of the total urinary and fecal excretion of urobilinogen may be used in human beings as an index of hemolysis. By this technique increases in the rate of hemolysis may be observed which might escape detection if routine clinical procedures only are used. It should be emphasized, however, that the total urinary and fecal excretion of urobilinogen must be determined if an index of the rate of blood destruction is to be obtained. Estimation of urinary urobilinogen alone does not yield this information since the greater portion of urobilinogen is normally excreted in the feces. Elevation of urinary urobilinogen values represents only the increased amount of pigment diverted from the feces to the urine, such as may occur in the presence of hepatic insufficiency or during very rapid hemolysis.

The content of urobilinogen in the feces and urine varies considerably

[1] Given at the Thirty-First Annual Meeting of the American Society for Clinical Investigation, Atlantic City, May 1, 1939.

in normal patients. Watson has stated that occasionally a normal individual may excrete as much as 250 mgm. of urobilinogen per day in the stools. Values as high as this were not encountered in the study of 26 normal individuals made in this laboratory. Total stool collections were made for 3- or 9-day periods and the urobilinogen output was found to vary from 75 mgm. to 150 mgm. a day. The urobilinogen excretion in the urine varied from 0.0 mgm. to less than 2 mgm. a day. These figures are in close agreement with those of Watson.

The present study deals primarily with the total urinary and fecal excretion of urobilinogen by 26 patients with pneumonia, of whom 20 were treated with sulfapyridine. The remaining 6 patients did not receive this drug.

Methods

Estimation of urobilinogen in stools and urine. The Watson-Terwen method (5) was used for the estimation of urinary and fecal urobilinogen. Stools and urine were collected over 3-day periods and kept in the icebox. Determination of the urobilinogen content was performed on the day following each 3-day period. Most of the patients were given milk of magnesia to ensure a daily defecation and occasionally tap water enemata were used. Diarrhea was not present in any instance.

Hematological studies. These studies were made on oxalated venous blood except in the case of stained films where capillary blood was used. Hemoglobin was estimated by the Sahli method. Red cell volumes were measured in Wintrobe tubes. The reticulocytes were counted in preparations stained supravitally with brilliant cresyl blue and counterstained with Wright's stain.

Liver function tests. Liver function studies were made in several patients by means of the bilirubin retention test of Harrop and Barron (6) and the sodium benzoate excretion test described by Quick (7).

Urinary studies. In addition to frequent routine urinalyses, in a number of instances determinations of kidney function were made by the urea clearance test of Møller, McIntosh and Van Slyke (8).

Sulfapyridine determinations. The sulfapyridine levels in blood and urine were estimated by a modification of the method used by Marshall and Litchfield (9) for the determination of sulfanilamide.

The patients on whom the studies were made were admitted to the hospital with a diagnosis of pneumonia. The diagnosis was confirmed in each case by physical and roentgenological examination, combined with careful studies of the sputum, blood, and exudates in order to determine the nature of the etiological agent.

Of the 26 patients, 18 suffered from pneumococcal pneumonia, and in 3 the disease was due respectively to *B. friedländeri*, a non-hemolytic streptococcus, and *Hemophilus influenzae.*

In 5 patients the etiological agent was not ascertained. Bacteriological examination did not reveal any microorganism which was considered of etiological importance in this group and the serial passages of throat washings and pleural exudates in mice and ferrets did not produce disease.[2]

Selection of patients was not made except in the case of the first 2 individuals, both of whom developed acute hemolytic anemia almost simultaneously at the beginning of the study.

Sulfapyridine was administered by mouth only. The dose varied from case to case depending upon the blood level of the drug and the duration of acute signs and symptoms of pneumonia.

The patients may be divided conveniently into three groups as indicated in Table I. The data are represented graphically in Figure 1.

Group I. Patients who did not receive sulfapyridine. None of the patients in Group I showed an excretion of urobilinogen in the stools above 166 mgm. per day. The highest urinary excretion was 3.3 mgm. Despite the presence of severe infection, in 3 patients the excretion of urobilinogen in the feces was 70 mgm. or less per day. In 4 of the 6 patients the etiology of the acute respiratory disease was not determined despite careful study. The other 2 had pneumococcal pneumonia.

Group II. Patients receiving sulfapyridine in whom excretion of urobilinogen was not increased. Ten of the 12 patients in Group II had pneumococcal pneumonia. In the remaining 2 the etiological agent was *B. friedländeri* and *H. influenzae*, respectively. The highest daily excretion of urobilinogen in the stools varied from 70 mgm. to 208 mgm.; the highest urinary excretion varied between 0.6 mgm. and 3.6 mgm. The total dosage of sulfapyridine varied between 4.5 and 27.0 grams, the average blood levels of the free drug between 1.7 mgm. per cent and 9.3 mgm. per cent. Two patients received unconcentrated Type III antipneumococcal rabbit serum, and 1 concentrated Type VI antipneumococcal rabbit serum. In 6 patients other disorders complicated the pneumonic process.

Group III. Patients receiving sulfapyridine in whom excretion of urobilinogen was increased. Of the 8 patients included in this group, 6 suffered from pneumococcal pneumonia. In 1 patient the etiological agent was not determined and in 1 pneumonia and empyema were due to a strain of nonhemolytic streptococcus. In the latter patient death occurred 2 months after the present studies were completed and was due to multiple brain abscesses which developed at a time when convalescence from the primary

[2] These studies were performed by Dr. Frank L. Horsfall, Jr., of the International Health Division of the Rockefeller Foundation.

TABLE I

Excretion of Urobilinogen in 26 Patients with Pneumonia, 20 of Whom Received Sulfapyridine

Case number	Age	Sex	Etiological agent	Sulfapyridine: Total dosage	Sulfapyridine: Duration of treatment	Sulfapyridine: Blood level during therapy: Highest	Sulfapyridine: Blood level during therapy: Average	Urobilinogen: Highest excretion per day: Feces	Urobilinogen: Highest excretion per day: Urine	Urobilinogen: Duration of increased excretion	Comment
				grams	*days*	*mgm. per cent*	*mgm. per cent*	*mgm.*	*mgm.*	*days*	
GROUP I											
1	63	M	Pneumococcus Type XXV					127	0.7		
2	24	F	Undetermined					64	0.61		
3	39	F	Undetermined					62	2.2		
4	22	F	Undetermined					166	3.3		
5	29	M	Undetermined					134	0.7		
6	20	M	Pneumococcus Type VIII					70	1.07		
GROUP II											
7	44	F	Pneumococcus Type I	17.0	3	11.1	9.3	80	1.3		Coarctation of aorta
8	57	M	B. friedländerii	27.0	7	10.4	6.7	206	1.6		Bronchiectasis
9	58	M	H. influenzae	18.0	4	2.2	1.7	208	3.6		Diabetes mellitus; empyema H. influenzae
10	5	M	Pneumococcus Type I	11.5	5	7.1	6.7	85	0.75		
11	57	M	Pneumococcus Type III	22.0	6			174	1.1		Pulmonary tuberculosis
12	66	F	Pneumococcus Type III	4.5	2			70	0.6		Auricular fibrillation; acute glossitis
13	75	M	Pneumococcus Type III	13.0	4	8.2	6.5	157	1.4		Received Type III rabbit serum
14	52	F	Pneumococcus Type IV	22.5	5	4.9	3.4	94	2.4		Chronic alcoholism
15	72	F	Pneumococcus Type VII	10.0	3	4.3	4.3	83	1.1		
16	73	F	Pneumococcus Type VI	8.0	4	4.2	4.1	120	1.0		Received Type VI rabbit serum
17	65	F	Pneumococcus Type III	14.0	4	4.6	3.1	127	1.3		Received Type III rabbit serum
18	15	M	Pneumococcus Type VII	18.5	4	3.1	2.7	56	1.1		

GROUP III

19	52	M	Pneumococcus Type III	78.0	10	19.6	11.0	891	4.0	14	Acute hemolytic anemia, received Type III rabbit serum; alcoholism
20	36	M	Pneumococcus Type II	81.0	11	7.7	5.8	561	0.99	10	Acute hemolytic anemia
21	59	F	Pneumococcus Type VI	18.0	4	4.5	3.0	272	0.42	3	
22	32	M	Undetermined	19.5	4	6.7	4.9	585	11.5	20	
23	41	M	Non-hemolytic streptococcus	12.0*	7			385	6.2	16	Empyema, died 2 months later of brain abscesses
24	52	F	Pneumococcus Type IX	24.0	8	3.9	3.7	452	1.6	7	Acute glossitis
25	18	M	Pneumococcus Type I	20.0	5	7.3	4.6	418	2.7	6	
26	26	F	Pneumococcus Type III	25.0	4	12.6	9.5	400	0.5	4	Received Type III rabbit serum. Acute azotemia; acute hemolytic anemia

* Received 10.0 grams of sulfanilamide in addition.

Group I = Patients who did not receive sulfapyridine.

Group II = Patients receiving sulfapyridine in whom excretion of urobilinogen was not increased.

Group III = Patients receiving sulfapyridine in whom excretion of urobilinogen was increased.

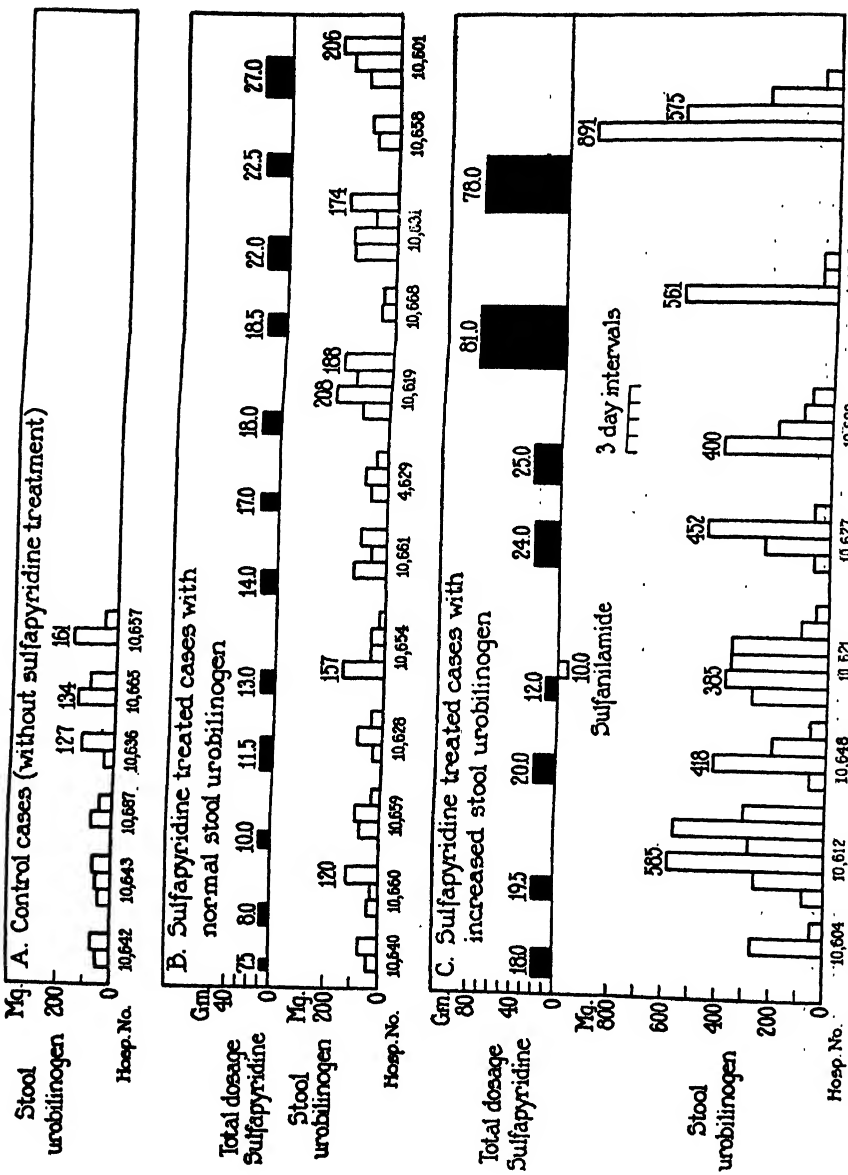

FIG. 1. Excretion of urobilinogen in 26 patients with pneumonia, 20 of whom received sulfapyridine

disease process was well established. Non-hemolytic streptococci were recovered from the abscesses.

The highest daily excretion of urobilinogen in patients' feces in Group III varied from 272 mgm. to 891 mgm.; these levels are well above those established as normal. The duration of increased urobilinogen output was from

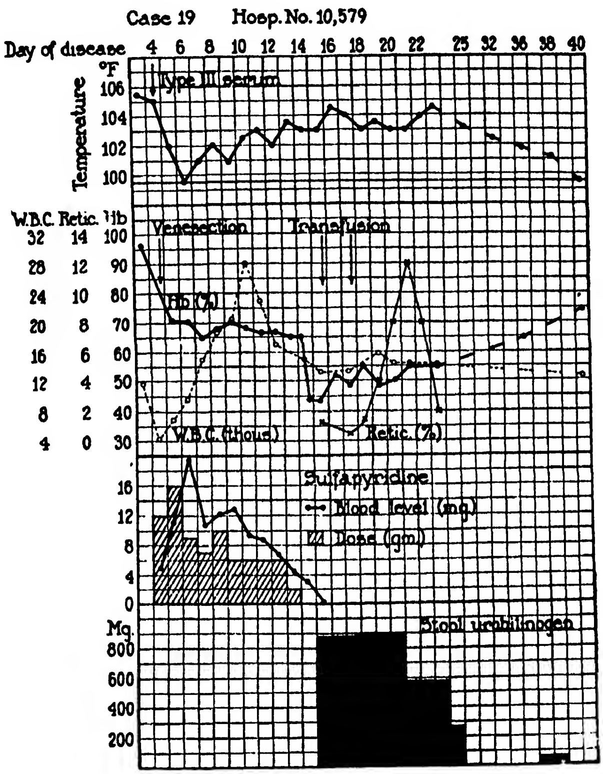

FIG. 2. Acute hemolytic anemia in patient with pneumonia treated with sulfapyridine

The temperature curve, blood changes, dosage and blood level of sulfapyridine, and excretion of urobilinogen in the feces are shown.

3 to 20 days. In 4 patients the increased excretion lasted for 10, 14, 16, and 20 days, respectively. The highest urinary urobilinogen excretion varied between 0.42 mgm. and 11.5 mgm. per day. The total dosage of sulfapyridine varied between 12.0 grams and 81.0 grams; however, the patient receiving the smallest dose was given 10.0 grams of sulfanilamide in addition. The average blood levels of free sulfapyridine were between 3.0 and 11.0 mgm. per cent. Two patients received unconcentrated Type III antipneumococcal rabbit serum as complementary treatment.

Three patients of this group developed acute hemolytic anemia. In 2, the total dosage of sulfapyridine was high—78.0 and 81.0 grams, respectively. In the third patient the output of urine diminished sharply the day after sulfapyridine therapy was begun. Acute azotemia occurred with the development of oliguria, and this was associated with high blood levels of sulfapyridine which were maintained for several days despite cessation of drug therapy. This patient developed a mild degree of hemorrhagic Bright's disease.

The case histories of the 3 patients who developed acute hemolytic anemia are briefly summarized.

CASE REPORTS

Case 19 (*Figure 2*). A white male, aged 52, was admitted to the hospital 32 hours after the acute onset of lobar pneumonia. His past history was non-contributory except for the excessive use of alcohol. On admission consolidation of the left upper lobe was present. Large numbers of Type III pneumococci were present in the sputum. Temperature was 104.6°, pulse rate 96, respiratory rate 26. The leukocyte count was 11,900, hemoglobin 96 per cent and the red blood cells numbered 5,060,000. Cultures of the blood showed no growth throughout the disease. Administration of unconcentrated Type III antipneumococcal rabbit serum was begun shortly after admission. Twenty-four hours later venesection was performed and 400 cc. of blood withdrawn because of impending pulmonary edema. The hemoglobin level dropped to 70 per cent following venesection. Although Type III agglutinins were present in the patient's serum in a titer of 1:256, the skin test with the Type III polysaccharide remained negative. On the second hospital day the pneumonic process spread to involve the right lower lobe. The leukocyte count declined to 4,900. Sulfapyridine administration was begun, a total of 80 grams being given over the succeeding 10 days. The blood level of free sulfapyridine was 19.05 mgm. per cent 48 hours after drug therapy was begun. The dosage of sulfapyridine was reduced during the succeeding 8 days, and on the day on which it was discontinued the blood sulfapyridine level was 3.4 mgm. per cent. During the course of drug therapy no extension of the pneumonic process occurred within the right lower lobe, but involvement of the entire left lower lobe took place and necessitated continuing the administration of sulfapyridine. The temperature remained irregularly elevated. The leukocyte count rose following administration of the drug, 28,000 being the highest count noted. No nausea or vomiting occurred, cyanosis was minimal, and little evidence of drug toxicity was apparent.

Administration of sulfapyridine was discontinued after 10 days. The hemoglobin level had fallen from 70 per cent to 60 per cent during the course of treatment. The following day a marked increase in pallor was observed. Blood examination showed: hemoglobin 44 per cent; red blood cells 1,910,000; white blood cells 14,550. The patient was transfused with 500 cc. of whole blood. Transfusion was repeated 2 days later because of continued hemolysis.

For the 3-day period during which the hemolytic process was at its height and before transfusion, the daily urinary urobilinogen output averaged 4.0 mgm. while the stool urobilinogen per day for the same period averaged 891.0 mgm. Liver function was

found to be within normal limits as measured by the sodium benzoate excretion and bilirubin retention tests. The urea clearance test reflected normal kidney function. There was a slight icteric tinge to the sclerae and the edge of the liver became palpable. In spite of the acute hemolytic process the reticulocyte count was below 2 per cent for the first 4 days after transfusion. A sharp rise to 12 per cent occurred during the succeeding

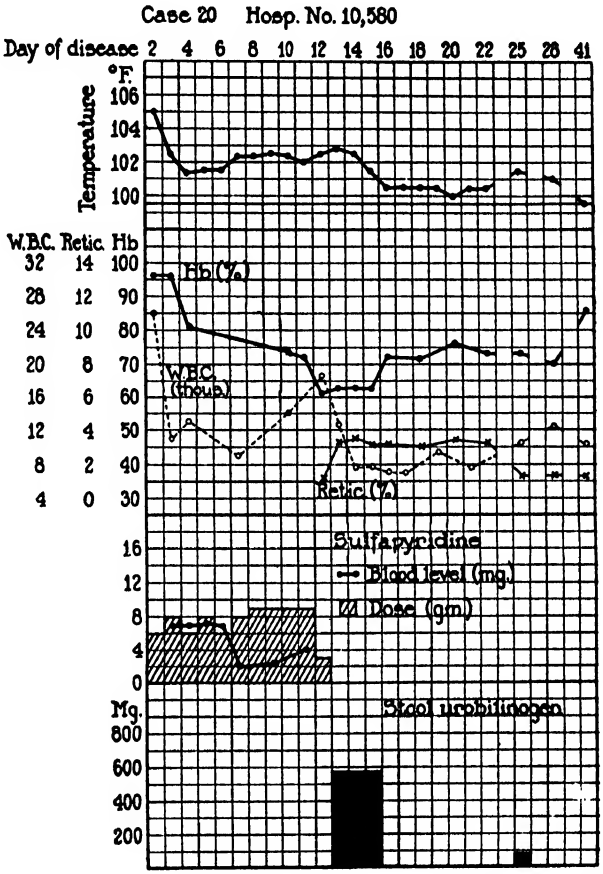

FIG. 3. Acute hemolytic anemia in patient with pneumonia treated with sulfapyridine

The temperature curve, blood changes, dosage and blood level of sulfapyridine, and excretion of urobilinogen in the feces are shown.

3 days, associated with a rise in hemoglobin to 55 per cent and of the hematocrit to 27. The daily urinary urobilinogen output averaged 1.4 mgm.; fecal urobilinogen 575 mgm. per day. Three days later the urobilinogen output in the urine had fallen to 0.37 mgm. per day and in the feces to 251.0 mgm. daily, indicating a marked decrease in blood destruction. Coincidentally, the hemoglobin level rose to 66 per cent, the hematocrit to 29. On discharge 6 weeks later the patient's hemoglobin level was 90 per cent; hematocrit 35; urobilinogen in the urine measured 0.82 mgm. daily and in the feces 63 mgm. daily.

Case 20 (*Figure 3*). A white male, aged 36, was admitted to the hospital 36 hours after the typical acute onset of lobar pneumonia. Temperature on admission was 104°, pulse rate 120, respiratory rate 38. Moderate cyanosis was present. The red blood cells numbered 4,650,000; hemoglobin percentage was 97, and the leukocyte count was 26,000. Consolidation of the left upper lobe was present. The sputum contained large numbers of Type II pneumococci. Blood cultures were sterile throughout the illness.

Treatment with sulfapyridine was begun 2 hours after admission and was continued for 11 days, a total of 81.0 grams being given. The blood level of sulfapyridine was determined frequently and found to vary considerably. The highest blood level recorded was 7.7 mgm. per cent of the free drug on the third day of treatment, and during the last 4 days the blood level varied between 2 and 4 mgm. per cent. Persistence of fever and acute signs and symptoms were associated with the development of a lung abscess in the left upper lobe, which became obvious as the surrounding acute pneumonic process resolved.

On the sixth day of drug therapy a fine macular skin rash appeared. This was confined mainly to the trunk and upper extremities and did not cause itching. The rash gradually faded and was not noted after the eleventh day.

During the course of treatment a moderate decline in hemoglobin values occurred, but on the eleventh day the patient was noticeably paler. The hemoglobin had fallen to 62 per cent and the red blood count to 3,040,000; white blood cells numbered 12,800; reticulocytes 3.2 per cent; hematocrit 29. At this time the average daily excretion of urobilinogen in the stool was 560 mgm. while that in the urine averaged 0.99 mgm. Transfusion was not considered necessary. The liver was not enlarged and there was no impairment of hepatic function, as measured by sodium benzoate excretion and bilirubin retention tests. Two weeks after the administration of the drug had been discontinued the hemoglobin level had risen to 74 per cent; red blood cells numbered 3,440,000; white blood cells 10,000; reticulocytes 1.4 per cent; hematocrit 31. At this time the urobilinogen excretion in the urine was 0.40 mgm. per day, and in the feces 74.5 mgm. Convalescence was much delayed due to the lung abscess which complicated the pneumonic process. Six weeks after admission the blood examination showed hemoglobin 86 per cent, red blood cells 4,320,000, white blood cells 10,300 and hematocrit 33.5.

Case 26 (*Figure 4*). A white female, aged 25, was admitted to the hospital on the eighth day following the onset of Type III pneumococcal pneumonia. Her past history was non-contributory. Consolidation of the right middle, right lower, and left upper pulmonary lobes was present. Temperature was 104.2°, pulse rate 132, respiratory rate 40. Blood cultures were sterile throughout the illness. Red blood cells numbered 3,790,000, hemoglobin level 74 per cent, leukocytes 21,450; urinalysis showed no abnormality; blood pressure was 112 systolic, 66 diastolic.

Twenty-five grams of sulfapyridine were given by mouth during the first 4 days following admission. Nausea and vomiting occurred and were fairly severe. The free sulfapyridine level in the blood was 12.6 mgm. per cent 36 hours after drug therapy was begun. This was associated with a marked diminution in the volume of urine. The patient's temperature and pulse rate fell to normal on the day following admission and agglutinins for pneumococcus Type III were demonstrable in her blood serum. The skin reaction to the Type III polysaccharide was positive at this time. Five days after

discontinuing the administration of sulfapyridine, the blood level of the free drug was 1.0 mgm. per cent, and in the urine the level was 12.4 mgm. per cent, indicating delayed excretion. The output of urine on this day was only 300 cc. The blood urea nitrogen was 61.0 mgm. per cent. Two days later the blood urea nitrogen level had fallen to 33.6 mgm. per cent and kidney function was 62 per cent of normal, as measured by the urea clearance test.

On the eleventh hospital day, urea clearance test showed 55 per cent of normal function; 1.4 grams of protein were excreted in the urine in 24 hours, and large numbers of granular casts were present. Centrifuged specimens showed only 2 to 4 red cells per high power field. The blood pressure was 162/92. Edema of the face and extremities was present.

During the 4 days on which sulfapyridine was given the urobilinogen excretion in the feces averaged 400 mgm. daily; in the urine 0.2 mgm. per day. The blood examination showed practically the same findings as on admission. During the 3 days immediately following the cessation of drug therapy, the urobilinogen excretion in the feces declined to an average of 196 mgm. daily; in the urine to 0.5 mgm. By the twelfth hospital day the red blood cell count had fallen to 2,000,000, with a hemoglobin of 50 per cent; the fecal urobilinogen excretion was 132 mgm. daily. A transfusion of 500 cc. of whole uncitrated blood was given. Two days later the red blood cells numbered 3,350,000, leukocytes 20,000, and the hemoglobin 66 per cent. Urobilinogen excretion in the feces continued to fall to a level of 114 mgm. daily.

Diminution in urinary output persisted for 20 days and was followed by a period of diuresis. Four weeks after onset of the renal complication kidney function had risen to 90 per cent of normal, as measured by the urea clearance test, but the urine continued to show a trace of albumin and a few granular casts for another month. On discharge 4 months after admission, the urea clearance test showed renal function to be 96 per cent of normal, and urinalysis showed no abnormality. Blood pressure was 128/78. The red blood cell count was 4,800,000; hemoglobin 96 per cent.

Cases 19 and 20 received large doses of sulfapyridine during periods of 10 and 11 days, the total dosage being 78 and 81 grams, respectively. Administration of the drug was prolonged in both instances because of the continuation of the acute disease; in Case 19 spread of the pneumonic consolidation occurred, and in Case 20 the acute process persisted in association with the development of a lung abscess. The blood levels of sulfapyridine are of interest in both of these patients. In Case 19 the maximum reached was 19.05 mgm. of the free drug per 100 cc. of blood on the third day of treatment. During the last 5 days of treatment the level declined from 12.6 mgm. per cent to 3.4 mgm. per cent. In Case 20 the highest blood level was 7.7 mgm. per cent and for the last 5 days of treatment it varied between 4.0 and 2.0 mgm. per cent. In Case 19 no signs of drug toxicity appeared other than acute hemolytic anemia. Despite the large dosage of sulfapyridine nausea did not occur, and there was no obvious increase in cyanosis. Case 20 suffered from severe nausea and vomiting during the

whole course of drug therapy and between the sixth and eleventh days of treatment a fairly generalized macular skin rash was present.

Case 26 received much less sulfapyridine than either of the above patients—25 grams over a 4-day period. However, abnormally high levels of the free drug occurred in the blood and the excretion was delayed in association with marked oliguria.

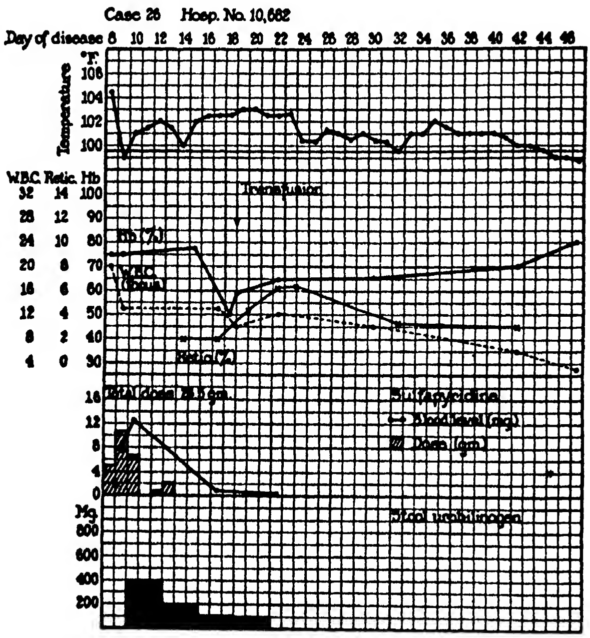

Fig. 4. Acute hemolytic anemia in patient with pneumonia treated with sulfapyridine

The temperature curve, blood changes, dosage and blood level of sulfapyridine, and excretion of urobilinogen in the feces are shown.

Case 19 showed the most severe anemia of the 3 and the highest daily excretion of urobilinogen in the stools. Likewise, the reticulocyte response was most marked in this case.

In all 3 patients who developed anemia the greatest depression of hemoglobin occurred about the twelfth day after the initial administration of sulfapyridine, regardless of dosage. The significance of this fact is not clear.

DISCUSSION

The widespread use of sulfapyridine in the treatment of pneumococcal pneumonia and other diseases has made a knowledge of its toxic effects important. The purpose of this communication is to present evidence

for the occurrence of increased hemolysis associated with the administration of sulfapyridine. An abnormally increased excretion of urobilinogen was noted in 8 of the 20 patients who received the drug and 3 of this group of 8 developed a severe degree of hemolytic anemia.

A correlation between the increased excretion of urobilinogen and the dosage of sulfapyridine cannot be made. However, in 2 of the patients who developed anemia a relatively high concentration of the drug in the blood was maintained for several days; in 1 by large oral dosages of sulfapyridine and in the second because of slow excretion presumably due to poor renal function.

The pneumonic process itself is apparently not responsible for increased erythrocyte destruction. The 6 patients of Group I who did not receive sulfapyridine, and the 12 of Group II who were treated with the drug, excreted normal amounts of urobilinogen even though suffering from acute febrile disease. However, the 8 patients of Group III who received the drug, excreted amounts of urobilinogen well above the limits of normal. Two patients in whom urobilinogen excretion was increased during the administration of sulfapyridine later excreted normal amounts when drug therapy was discontinued, even though acute febrile disease persisted.

The reason for increased hemolysis incident to the administration of sulfonamide compounds is not known. It is possible that certain patients vary in their susceptibility either to the sulfonamide compound itself or to one of the derivatives formed within the body. It is likewise possible that certain patients may convert more of the sulfonamide compound into hemolytic products than others, or else fail to detoxify and eliminate these products rapidly enough to prevent increased hemolysis.

SUMMARY

The excretion of urobilinogen in feces and urine has been measured in 26 patients with pneumonia, 20 of whom received sulfapyridine.

In 18 patients the excretion of urobilinogen was within normal limits. Twelve patients were treated with sulfapyridine and 6 did not receive the drug.

Eight patients who received sulfapyridine excreted increased amounts of urobilinogen. Hemolytic anemia occurred in 3 of these.

BIBLIOGRAPHY

1. *a.* Wood, W. B., Jr., Anemia during sulfanilamide therapy. J. A. M. A., 1938, **111**, 1916.

b. Antopol, W., Applebaum, I., and Goldman, L., Two cases of acute hemolytic

anemia with autoagglutination following sulfanilamide therapy. J. A. M. A., 1939, **113**, 488.

c. Koletsky, S., Fatal hemolytic anemia following the administration of sulfanilamide. J. A. M. A., 1939, **113**, 291.

d. Flippin, H. F., Lockwood, J. S., Pepper, D. S., and Schwartz, L., The treatment of pneumococcic pneumonia with sulfapyridine; progress report on observations in 100 cases. J. A. M. A., 1939, **112**, 529.

e. Long, P. H., and Wood, W. B., Jr., Observations upon the experimental and clinical use of sulfapyridine. II. The treatment of pneumococcal pneumonia with sulfapyridine. Ann. Int. Med., 1939, **13**, 487.

2. Brownlee, G., The rôle of the aromatic amino group in deranged pigment metabolism. Biochem J., 1939, **33**, 697.
3. Rimington, C., Disturbances of pigment metabolism following administration of drugs of the sulphonamide series and simpler related substances. Proc. Roy. Soc. Med., 1939, **32**, 351.
4. Rimington, C., and Hemmings, A. W., Porphyrinuric action of drugs related to sulphanilamide. Biochem. J., 1939, **33**, 960.
5. Watson, C. J., Studies of urobilinogen; improved method for quantitative estimation of urobilinogen in urine and feces. Am. J. Clin. Path., 1936, **6**, 458.
6. Harrop, G. A., and Barron, E. S. G., The excretion of intravenously injected bilirubin as a test of liver function. J. Clin. Invest., 1931, **9**, 577.
7. Quick, A. J., The synthesis of hippuric acid: a new test of liver function. Am. J. M. Sc., 1933, **185**, 630.
8. *a.* Møller, E., McIntosh, J. F., and Van Slyke, D. D., Studies of urea excretion; relationship between urine volume and rate of urea excretion by normal adults. J. Clin. Invest., 1928, **6**, 427.

b. McIntosh, J. F., Møller, E., and Van Slyke, D. D., Studies of urea excretion; influence of body size on urea output. J. Clin. Invest., 1928, **6**, 467.

9. Marshall, E. K., and Litchfield, J. T., Jr., Determination of sulfanilamide. Science, 1938, **88**, 85.

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SWINE POX

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To judge from the literature, there is much disagreement as to the proper allocation of the virus of swine pox within the group of the animal pox viruses. The works of *Bollinger*[1], *Koch*[2], *Peiper*[3], *Gins*[4], and *Akazawa* and *Matsumura*[5] indicate a close relationship between swine pox virus and vaccinia virus. *Bollinger*[1] believed the swine virus to be derived from either cow or sheep pox. He did not consider swine pox to be a disease *sui generis* because it was so seldom encountered under natural conditions. *Koch*[2] was able to transmit swine pox virus to calves. *Peiper*[3], whose information apparently came from *Bollinger*, contended that swine pox was transmissible to man and produced lesions like those caused by vaccinia virus. *Gins*[4] was able to lapinize the strain of swine pox virus he studied in 4 rabbit passages and it then produced characteristic vaccinal lesions on a calf. *Akazawa* and *Matsumura*[5] were also successful in lapinizing their strain of the swine pox virus.

The experiments of *Velu*[6] and of *Zabala*, *Maggio*, and *Rosenbusch*[7], however, fail to support the view that swine pox virus stems from vaccinia virus or in fact is even closely related to it. *Velu*[6] found no evidence from his observations in Morocco that swine pox was associated with variola or vaccinia. Furthermore, an attack of swine pox failed to immunize pigs against vaccinia virus. *Zabala*, *Maggio*, and *Rosenbusch*[7] attempted to test the cross-immunity in the reverse direction and found that inoculation with vaccinia virus did not immunize pigs against swine pox virus. However, since the vaccinia virus they employed failed to produce lesions in swine their observation is of doubtful significance. *McNutt*, *Murray*, and *Purwin*[8] state that the swine pox virus they studied failed to infect rabbits, although they mention no attempts to lapinize it by serial passage.

The present experiments were undertaken with the purposes in mind, first, of studying a North American strain of swine pox virus more thoroughly than one had heretofore been studied, and second, of learning whether this virus was indeed a specific infective agent of swine or whether it was merely vaccinia virus in swine.

EXPERIMENTAL

Source of Swine Pox Virus Studied.—Swine pox is a very prevalent and widespread disease in the United States and is frequently encountered not only in swine droves in the middle western states but in the eastern states as well. The disease is usually mild and unaccompanied by fatalities. Occasionally, however, for reasons that are not apparent, outbreaks in certain droves are more serious, and from 5 to 30 per cent of the animals in the drove may die. The virus employed in the present experiments came from a relatively benign outbreak of swine pox among swine on a farm in southeastern Iowa.* The virus was collected as dried scabs and was brought back to the laboratory in 50 per cent glycerol.

Experimental Transmission.—No difficulty was encountered in transmitting swine pox experimentally to swine. Areas of skin on the swine to be infected were scarified with a surgical needle and immediately thereafter suspensions of the virus were applied and rubbed in with the handle of a scalpel. Virus consisted in the supernatant of 2 to 5 per cent suspensions of scabs or pustules in physiological saline.

Clinical Features of Experimental Swine Pox

The local lesions induced by the virus are quite regular and constant. After an incubation period usually of 5 to 7 days but rarely as long as 12 days, reddened hyperemic papules from 3 to 7 mm. in diameter appear along the scarifications. These papules persist relatively unchanged for 2 or 3 days and then rather abruptly the apices of the papules acquire the appearance of pustules. Occasionally a very transient vesicle stage is to be observed. The papulo-pustules have a slightly greater diameter than the papules preceding them. The lesions progressively become more pustular and within another 2 days acquire the appearance of true pustules (Fig. 1). At this stage the lesions are frequently umbilicated. The pustules persist for 6 to 8 days at the end of which time they become lightly and superficially scabbed. The scabs become progressively thicker and heavier and are dark brown to black in color. They persist for a long time, frequently for 2 or 3 weeks, and finally are shed, usually without noticeable scarring.

No secondary lesions occur in louse-free pigs. However, in swine that are infested with hog lice (*Haematopinus suis*) numerous secondary lesions appear, especially in the inguinal and axillary regions, on the 8th to the 14th

* I am indebted to Dr. *R. F. Miller* and Dr. *R. M. Hofferd* for making this material available to me.

day after the first appearance of lesions on inoculated areas. From subsequent investigation of the rôle played by the hog louse in transmitting swine pox virus the secondary lesions are considered to result from transport of virus from the primary inoculation to other areas of skin by the louse. The rôle played by lice in swine pox will be more fully discussed later.

The general manifestations of illness in experimental swine pox are more variable than the course of the local lesions. The majority of the animals do not appear definitely ill and exhibit no elevation of temperature. However, occasional animals have become quite ill with depression, anorexia,

FIG. 1. Swine pox transmitted experimentally by hog lice. The lesions are 9 days old and are in the pustule stage. They tend to be discrete over most of the body but are confluent in the axillae, groin, and on the ears, sites of predilection for lice. Photographed by Julian A. Carlile.

and high fever as the cardinal signs of their illness. Usually these have been animals in which skin lesions were most extensive and widespread.

The clinical picture outlined thus far is that exhibited by swine inoculated by applying virus to scarified skin. In 3 animals inoculated with relatively large doses of virus (5 c. cm. of a 2 per cent suspension) into the blood stream the picture was that of a more serious illness. The incubation period in all 3 swine was either 13 or 14 days. The animals appeared ill and exhibited elevated temperatures a day before lesions appeared. The lesions, which were thick-set and diffusely scattered over the entire body, progressed through the same stages as those following scarification but their course was more rapid. The papule stage lasted for only 2 days and then only an additional period of 4 to 6 days of pustules elapsed before the appearance

of scabbing lesions. The animals became progressively more ill as the disease advanced. Their temperatures were elevated throughout, ranging from 40°C. to 41,5°C.; their appetites, diminished during the early course of the disease, were abolished after the 5th day; they became progressively more depressed and all were prostrate by the 7th day. It is believed that

FIG. 2. Section of early swine pox lesion (4th day) showing dermis densely infiltrated with many lymphocytes and few polymorphonuclear leucocytes. The epidermis is thickened and sparsely infiltrated with leucocytes. Vacuolation of the cytoplasm of individual epidermal cells is apparent. Hematoxylin-eosin. × 115. Photographed by Julian A. Carlile.

all 3 would eventually have succumbed of the disease. They were sacrificed on the 9th or 10th days and at this time were literally plastered with scabs and extremely ill.

Pathology

Gross pathological alterations are limited to the skin and the inguinal lymph nodes. The early skin lesions consist, as has been mentioned, in

FIG. 3. Higher power of section of early swine pox lesion (4th day) showing vacuolated epidermal cells. Granular acidophilic inclusions may be seen in the vacuolated cytoplasm of many of the cells. Hematoxylin-eosin. × 295. Photographed by Julian A. Carlile.

multiple red papules. These later progress to pustules and finally become scabbed before healing. In swine in which lesions are numerous on the skin of the lower abdomen, groin, and thighs, the inguinal lymph nodes become greatly hypertrophied and readily palpable. They are firm and lobulated and there is usually some surrounding subcutaneous edema.

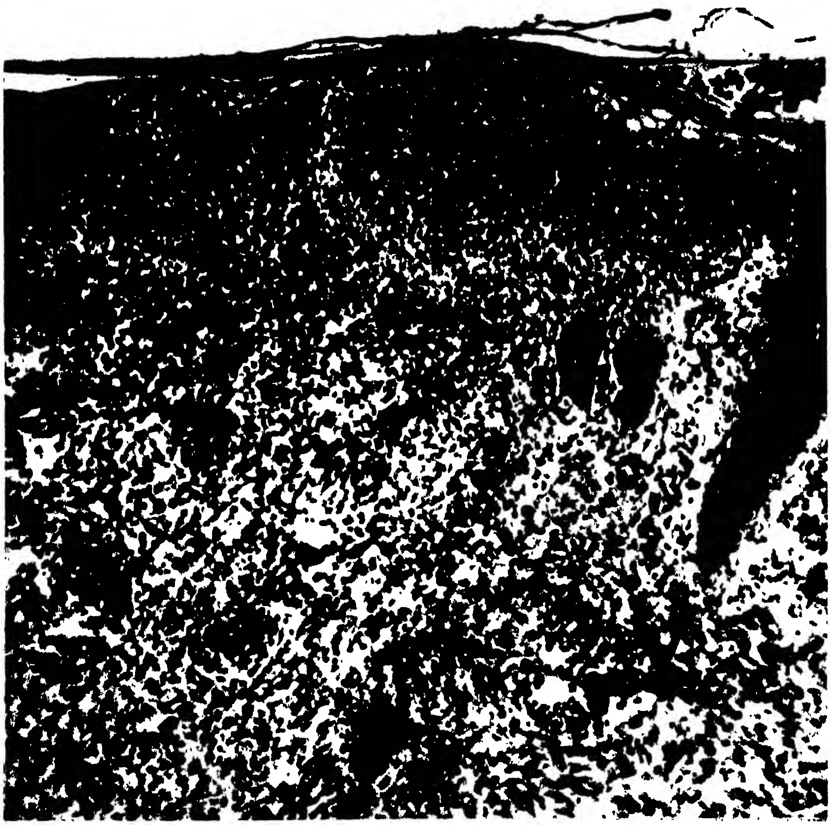

FIG. 4. Section of later swine pox lesion (9th day) showing dense infiltration of epidermis by polymorphonuclear leucocytes. The gross appearance of this lesion was that of a pustule. Hematoxylin-eosin. × 115. Photographed by Julian A. Carlile.

They are not congested. They cut almost as though they were fibrous and there is usually a copious oozing of clear fluid from the cut surface which is a uniform pinkish-white in color. There are no evident areas of necrosis or caseation.

Histologically the skin lesions of swine pox involve both the epidermis and the dermis. Sections of early lesions still in the papule stage show myriads of inflammatory cells infiltrating the dermis (Fig. 2). These

are largely lymphocytes, although some polymorphonuclear leucocytes are also present. The overlying epidermis is thickened, and sparsely infiltrated with polymorphonuclear leucocytes, both neutrophiles and eosinophiles. The most striking alteration in the epidermis, however, consists in widespread cytoplasmic vacuolation of the epidermal cells (Fig. 3). This is most marked in the superficial cell layers but in places it extends almost down to the dermis. Affected cells are considerably enlarged, their nuclei may or may not stain poorly, and in their vacuolated cytoplasm are from one to several acidophilic granular inclusions. In later stages, when the gross lesion is that of a pustule, the dermal infiltration becomes more intense with lymphocytes and polymorphonuclear leucocytes participating about equally (Fig. 4). The epidermis in this stage is diffusely infiltrated with polymorphonuclear leucocytes, largely eosinophiles.

Sections of involved inguinal lymph nodes show little of significance aside from a diffuse lymphoid hyperplasia. The germinal follicles appear active and there is considerable intercellular edema. In some of the more advanced cases accumulations of eosinophiles are present in some of the lymph sinuses. The significance of the presence of large numbers of eosinophiles both in the lymph nodes and in the skin lesions is not known, but they are present irrespective of whether the animal supplying the tissues was louse-infested or not.

Characters of the Virus

The infective agent of the strain of swine pox under discussion is a virus of the elementary body type. Suspensions prepared from scrapings of early lesions and treated by the silver impregnation method of *Morosow* contain myriads of elementary bodies identical in appearance and size with those of similar preparations of vaccinia lesions. Probably because of its relatively large size, the virus has not proved filtrable through Berkefeld candles.

The virus is relatively resistant and is capable of survival for long periods of time if kept under appropriate conditions. Strips of infected skin have yielded infective virus after storage for a year in 50 per cent glycerol in the refrigerator. There has been no occasion to test its survival for longer periods.

The virus has consistently failed to infect rabbits when injected testicularly or when applied to the scarified skin and did not lapinize in four "blind" serial testicular or cutaneous rabbit passages. Its presence, however, could be demonstrated, by swine inoculation, in the testicle of a rabbit 8 days

after injection. The virus failed to produce lesions in the scarified skin of a calf to which it was administered.

Distribution of the Virus in Infected Swine

Virus has proved abundantly present in the early skin lesions and is demonstrable in dried scabs for at least 16 days after lesions have first appeared. It is present in low concentration in superficial lymph nodes draining areas of infected skin but has not been found in the blood stream nor in the liver and spleen on repeated test.

Immunity

One attack of swine pox renders swine solidly resistant to reinfection. However, although recovered animals are immune, their sera either fail completely to neutralize the virus or neutralize it only in very low titer. No serum from a recovered swine has been found capable of neutralizing more than 10 minimal infective doses of the virus, and most such sera are devoid of demonstrable virus-neutralizing antibodies.

Hog Lice in the Transmission of Swine Pox Virus

In an effort to determine whether the swine pox strain under investigation would transmit by contact, normal pigs were placed in the same pens with infected animals. It was found that if the swine were free of hog lice no transmission from the sick to the well animals occurred. However, if the animals were louse-infested, infection of the normal animals regularly occurred after an incubation period of 12 to 18 days. This suggested that the hog louse was capable of serving as an intermediate host for swine pox virus. On subsequent search of the literature it was found that *Schang*[9] and *Csontos* and *Nyiredy*[10] had already made a similar observation. The present findings confirm their earlier observations.

Swine pox virus could be demonstrated in or on lice from infected swine either by permitting such lice to feed on normal swine or by applying saline suspensions of the lice to the scarified skin of normal swine. Lice from infected swine were tested for infectivity at various intervals after the first appearance of lesions on their source hosts. Virus was demonstrable on repeated occasions in lice removed on the 4th and 12th days after the first appearance of lesions, once on the 15th day; but single tests of lice removed on the 19th, 22nd, and 24th days proved negative despite the persistence of scabbed lesions on the source hosts. It would appear from these observations that little or no multiplication of the virus takes place within the louse, that the virus does not even persist for long within the

louse, and that the louse probably serves more as a mechanical vector, in transmitting the virus, than as a true intermediate host. Efforts to determine the period of survival of the virus in lice removed from infected swine and kept in vials without feeding were handicapped by the rapidity with which these lice succumbed when not permitted access to their natural host. They transmitted swine pox when fed on normal swine 4 and 7 hours after removal from infected animals but were dead by the time the next proposed feeding was due.

Absence of Relationship between Swine Pox and Vaccinia Viruses

There is no doubt that swine are naturally susceptible to vaccinia and that they respond to the presence of virus in the skin with a vesicular reaction. *Nelson*[11] used the New York City Department of Health strain of vaccine in his work with the stock of swine employed in the present experiments and has described the clinical course of the vaccinal lesions observed. The incubation period is short and generally by the 2nd day slight elevation of the skin with a little congestion may be observed along the inoculated scarifications. Definite papules, discrete or confluent, develop on the 3rd or 4th day and a day later these become vesicular. The following day scab formation is present. Immediately after scabbing the swelling rapidly subsides, the congestion fades, and the lesions as a whole regress rapidly.

Our experience with the same strain of vaccinia virus* in swine corresponds with that of *Nelson*. It is thus evident that the course of vaccine infection in swine is much more rapid than that of swine pox. Furthermore, the lesions of vaccinia, though resembling those of swine pox, are more superficial and persist for only a fraction of the time that the swine pox lesions remain.

The absence or low titer of swine pox virus-neutralizing antibodies in sera of pox-recovered swine, as well as the failure of swine pox virus to adapt to rabbits, handicapped a serological comparison of swine pox virus with vaccinia virus. It was necessary to test the swine pox virus-serum mixtures for neutrality in swine while the vaccinia virus-serum mixtures were tested for neutrality in rabbits. As stated earlier, swine pox-convalescent serum either failed to neutralize or neutralized swine pox virus poorly: it failed to neutralize vaccinia virus. In the reverse direction,

* The vaccinia virus employed in this work was obtained from the Laboratories of the New York City Department of Health through the courtesy of Dr. *Ralph S. Muckenfuss*.

vaccinia-convalescent serum, though neutralizing vaccinia virus in high titer, was without effect upon swine pox virus. The experiments thus furnished no evidence of a serological relationship between the viruses of swine pox and vaccinia.

In order to determine whether either vaccinia or swine pox virus would induce an immunity for the heterologous virus, 2 swine were infected with vaccinia virus and 2 others with swine pox virus. Both pairs of animals reacted typically to infection. After complete recovery in the case of the vaccinia animals and after scabbing and beginning regression of the lesions in the case of the swine pox animals, all four of the pigs were inoculated simultaneously with both vaccinia and swine pox viruses at separate sites. The 2 animals that were recovered from vaccinia developed typical lesions of swine pox after the regular incubation period; but they failed to develop vaccinia lesions. Conversely the two animals that were convalescing from swine pox developed typical vaccinia lesions but proved refractory to swine pox virus. There was thus no evidence that previous infection with either vaccinia or swine pox virus exerted the slightest influence upon subsequent infection with the heterologous virus. It is apparent from the observations described that the swine pox and vaccinia viruses studied in the present investigation are distinct and different infective agents and are probably totally unrelated to one another.

DISCUSSION

The observation that swine pox virus is distinct from and probably unrelated to vaccinia virus is in agreement with the minority of earlier workers who have considered the question of relationship. The discrepancy between the two groups of workers, those maintaining no relationship and those maintaining a relationship between the two viruses, is not, however, entirely without plausible explanation if one admits that vaccinia virus may at times cause a natural infection in swine. In such a case the type of disease resulting would probably resemble that produced experimentally in swine, would have a shorter incubation period, and would run a more rapid course than the swine pox studied in the present investigation. However, the lesions seen in these vaccinia-infected pigs could certainly be designated as pocks and it would thus be permissible to refer to the disease itself as swine pox. Investigators obtaining their source material from such outbreaks would find upon studying it that the causative agent was vaccinia virus and would consequently record their observations as indicating that the causative agent of swine pox was indeed vaccinia

virus. On the other hand, investigators who happened to obtain their source material from an outbreak such as the one furnishing the virus used in the present study, would conclude upon equally permissible grounds that the causative agent of swine pox was distinct from vaccinia virus and was indeed not even antigenically related to that virus. Upon such an interpretation of the experimental discrepancies evident from reports in the literature it seems apparent that at least two etiologically distinct conditions are diagnosed in the field as swine pox. One of these is a vaccinia virus infection in swine, and the other is a pock disease caused by an agent of the type studied in the present experiments, apparently totally unrelated to vaccinia virus. This latter agent, it would seem, bears a just claim to being designated the swine pox virus.

From a practical standpoint, the control of swine pox, as a field disease, would seem to depend largely upon adequate care to prevent infestation with hog lice. In the absence of lice no spread of the disease from animal to animal takes place even under the conditions of close pen contact, while among louse-infested swine it spreads with great facility.

SUMMARY

1. The clinical and pathological pictures produced in experimental swine by a strain of swine pox virus obtained in Iowa are described.

2. The infective agent is a virus of the elementary body type and is capable of storage in glycerol for relatively long periods of time. It is not pathogenic for rabbits and failed to lapinize in four "blind" serial passages in rabbits.

3. One attack of swine pox renders pigs solidly immune to reinfection, but the presence of virus-neutralizing antibodies in the sera of recovered swine is quite irregular.

4. Hog lice are capable of transmitting swine pox virus from animal to animal but probably serve more as mechanical vectors than as true intermediate hosts.

5. Vaccinia virus and the strain of swine pox virus studied in this investigation are not immunologically related.

6. It is suggested that discrepancies in the literature concerning the relationship between vaccinia and swine pox viruses may indicate the existence in swine under field conditions of two distinct pock diseases. One of these diseases is probably caused by infection with vaccinia virus while the other is caused by infection with a virus of the type studied in the present investigation.

BIBLIOGRAPHY

[1] *Bollinger, O.:* Slg. klin. Vortr. (Innere Medizin Nr. 24, S. 1021). Leipzig: R. Volkmann, 1877.—[2] *Koch:* Öst. Mschr. Tierheilk. Nr. 8, 57 (1887).—[3] *Peiper, E.:* Die Pockenimpfung, S. 20. Berlin u. Wien: Urban & Schwarzenberg, 1901.—[4] *Gins, H. A.:* Z. Hyg. usw. **89:** 231 (1919).—[5] *Akazawa, S.* and *T. Matsumura:* J. Jap. Soc. of Vet. Sci. **14:** Nr. 1 (1935).—[6] *Velu:* Rec. méd. vét. **92:** 24, 28 (1916).—[7] *Zabala, J., C. Maggio,* and *F. Rosenbusch:* Viruela de los Cerdos. Buenos Aires: Flaiban and Camilloni, 1917.—[8] *McNutt, S. H., C. Murray,* and *P. Purwin:* J. amer. veterin. med. Assoc. **74:** 752 (1929).—[9] *Schang, P. J.:* Rev. Med. Vet. **15:** 90 (1933).—[10] *Csontos, I.* and *S. von Nyiredy:* Dtsch. tierärztl. Wschr. **41:** 529 (1933).—[11] *Nelson, J. B.:* J. Exper. Med. (Am.) **56:** 835 (1932).

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THE BACTERIA-FREE CULTURE OF A NEMATODE PARASITE

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It has been possible for some time to grow the entire life cycle of *Neoaplectana glaseri*,[1] a nematode parasite of the Japanese beetle, in cultures in which bacterial and fungous growths have been inhibited in various ways but not eliminated.[2] These contaminants undoubtedly introduced a high degree of variability into the results obtained. It therefore seemed advisable to attempt to rear this parasite in cultures free from bacteria.

Lapage[3] and Glaser and Stoll[4] developed technics whereby the second ecdysis of strongyloid nematode larvae was easily and consistently obtained in quantity under sterile conditions. It was found necessary to modify one of these technics slightly for work with *Neoaplectana*. Cultures prepared in the routine manner were permitted to develop for 10 to 15 days, at which time the majority of the parasites were second-stage larval forms in their third or fourth generation.* These were removed from the surface of the solid medium and washed with water until free of much débris. To remove the dead larvae they were then filtered through 2 layers of lens paper supported by fine gauze. The larvae were ensheathed by aerating them in 25 cc of water for 3 to 4 days with a change of clean water 3 times daily. They were then washed in 25 cc lots of sterile water 3 times each day for 3 days, after which they were treated for 30 minutes to 1 hour with Labarraque's solution (sodium hypochlorite) at a dilution of 1 part to 40 or 50 parts of water. The nemas were again washed 3 times in sterile water, and then placed in water about 5 mm deep for from 15 to 20 hours.†

[1] Steiner, G., *J. Washington Acad. Sci.*, 1929, **19**, 436.

[2] Glaser, R. W., *Science*, 1931, **73**, 614; Circ. 211, Dept. of Agric., State of New Jersey, 1932; *Studies from The Rockefeller Institute for Medical Research*, 1932, **83**, 521; McCoy, E. E., and Glaser, R. W., Circ. 265, Dept. of Agric., State of New Jersey, 1936; McCoy, E. E., and Girth, H. B., Circ. 285, Dept. of Agric., State of New Jersey, 1938.

[3] Lapage, G., *J. Helm.*, 1935, **13**, 103; *Parasitology*, 1935, **27**, 186.

[4] Glaser, R. W., and Stoll, Norman R., *J. Parasit.*, 1940, **26**, 87.

* The ensheathed second-stage larva is the only stage capable of surviving free in nature and represents the invasive form which must penetrate into a host (Japanese beetle grub) to continue its development.

† During this time interval *Neoaplectana* digested and evacuated the greater part of its intestinal flora.

The next day the nemas were again treated with Labarraque's solution and then washed 3 times, followed by another 15 to 20 hours' sojourn in a small amount of water. This was followed by a third treatment with Labarraque's solution and 3 more washings with sterile water. Finally, the nemas were again passed through sterile lens paper, to remove any worms that had died during the manipulations, and the viable forms were stored in shallow water until used.

When the above procedures were carefully followed sterile larvae were obtained, shown by the fact that no bacterial growth occurred when they were cultured on standard laboratory media under both aerobic and anaerobic conditions. When occasional contaminants appeared later after a prolonged incubation period, such cultures were either placed aside for collateral observations or discarded.

At first it was thought necessary to have a solid substrate to facilitate the movements and ecdyses of *Neoaplectana*. Neutral veal infusion agar slants may be used, but a simple substrate of 2% agar prepared with 0.5% sodium chloride solution answered just as well. Ten cc of the melted agar were slanted in culture tubes measuring 180 x 22 mm. About one gram of animal tissue, removed under sterile conditions, was then placed at the base of the slant, and 2 or 3 drops of sterile 0.5% salt solution were added. Each tube was inoculated with ±200 of the previously sterilized second-stage larvae and then sealed by pouring melted sealing wax over the cotton stopper previously trimmed and pushed down into the tube for approximately half an inch. When hard the sealing wax was perforated by a hot wire. Sealing in this manner prevents excessive evaporation without excluding oxygen. Recently we have also sealed many tubes with "Parafilm" perforated with a few needle pricks and have found this satisfactory. The sealed tubes were held in a slanted position and incubated at room temperatures, 22–28°C.

Eighteen- to 20-day-old mouse embryo, beef kidney, and rabbit ovary and kidney have all been found to support growth. The last proved to be the easiest to manipulate and to give the best growth. Consequently it has been used for most of the work. In tubes containing approximately one gram of rabbit kidney the tissue is almost completely digested in from 18 to 24 days; at this same period of incubation, growth has reached its maximum and second-stage larvae predominate. Cultures may be held for at least 3 months without transplantation, but to maintain vigorous growth, transfers are made every 3 weeks. Sterile nematodes have to date been maintained without loss through 14 transfers or approximately 50 generations. With the methods previously used it was necessary to add

certain accessory growth factors in order to maintain the cultures longer than 21 to 32 generations.[5] The nematodes have also been cultured in Erlenmeyer flasks containing 0.5% NaCl solution to a depth of 3 to 4 mm in which are placed pieces of sterile rabbit kidney, and in liquid media containing kidney extracts devoid of particulate matter.

On agar slants containing rabbit kidney the number of nematodes found after incubation for 18 days is approximately 150,000. When grown on Petri dishes, 5.5 cm in diameter, containing 2% dextrose agar and living yeast, the yield was about 40,000. Contamination of the rabbit kidney causes a marked reduction, and in some cases an absence, of growth.

In view of the fact that this parasitic nematode can be grown on both liquid and solid media free from bacteria it will be possible to study its nutritional and other requirements and to test the effects of various vitamins and hormones.

[5] Glaser, R. W., *J. Exp. Zool.*, in press.

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CONTINUED CULTURE OF A NEMATODE PARASITIC IN THE JAPANESE BEETLE[1]

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INTRODUCTION

In 1931 and more fully in 1932, the writer reported the culture of the entire life cycle of Neoaplectana glaseri, an ovoviviparous nematode parasite of the Japanese beetle, Popillia japonica. The development of the different stages of this roundworm was obtained on a 2% dextrose-veal infusion agar with living yeast. The yeast cells, by forming a mat on the surface of the medium, suppressed bacterial growth and also furnished nutriment. Under these conditions a new generation appeared every 4 or 5 days and within about 2 weeks three to four generations had occurred in each culture. A few cultures survived the tenth or eleventh transplant, lasting about 20 weeks, but most of the nematodes waned and died after the seventh or eighth transplant or in 14 to 16 weeks, during which time 21 to 28 or 24 to 32 generations, respectively, were produced. It was difficult to determine whether a culture was going to survive after the seventh or after the eighth transplant. It sometimes began to wane after the sixth and subsequently died; on the other hand, heavy cultures after the sixth transplant often disappeared completely following the seventh. At first, cultures could not be prevented from waning, and to continue the study it was necessary again to find parasitized grubs in the field. Later, it was found that when weakening cultures were passed through healthy grubs a number of times, they could be saved from extinction. The parasites recovered after passage again survived the seventh or eighth transplant on the medium described above.

Examination showed that the cultures became extinct because no more young were produced. Both males and females appeared normal in size and shape, but in the females the ovaries failed to mature and consequently ova were not formed. The male sex glands were apparently unaffected. A further investigation was instituted in an attempt to clarify this situation and the results are reported in the present paper.

[1] Conducted cooperatively by The Rockefeller Institute for Medical Research, Princeton, New Jersey, and the New Jersey Department of Agriculture.

GENERAL PROCEDURES

In all experiments veal infusion agar containing 2% dextrose was used as a basic medium. Small Petri dishes measuring 5½ cm. in diameter were employed throughout, and into these 10 cc. of the melted nutrient agar were poured. When yeast or the natural flora from the nematodes was desired, this was permitted to develop on the surface of the agar for 24 hours prior to inoculation with the parasites. Throughout the culture period the surface of the medium was kept moist with sterile, distilled water. Good development was obtained when the medium initially reacted pH 5.0 to 9.0. After nematode development ceased the final reaction of the surface fluid or of the re-melted agar was always alkaline, varying from pH 8.2 to 9.0. An initial reaction of pH 7.3 was chosen for the tests, and alkaline stability was always reached in 10 to 15 days with a mean of 11 days.

For certain tests various substances were sprinkled on the surface of the medium. For all powders the amount equalled 0.02 gm. after preliminary work showed that more or less than this had no further effect. When fluids or materials in suspension were used the amounts will be indicated later under specific experiments.

When the medium was ready the nemas were obtained from a parasitized grub and placed in two changes of water. Here the coarse débris was removed with a pipette. The nemas were then washed three times in three changes of water, after which they were placed on the medium. This same cleaning process was observed at the time of each transplant. Unless otherwise stated, each culture plate received about 1000 second-stage forms by means of a calibrated pipette filled with a uniform suspension of the nemas. When inoculated as above, Neoaplectana cultures reach their maximum development on the tenth to the twelfth day at which time most of the worms will be second-stage forms in their third generation. Many observations showed that 10 days was the mean time when the maximum culture yield might be expected. This equalled about 40,000 nemas for a 5½ cm. dish containing the standard mixture of dextrose-veal infusion agar and living yeast. Some of the results to be presented were therefore based on this 10-day period; the tenth day, incidentally, also represented the time of transplantation.

For certain experiments separate strains of Neoaplectana were used. In the sense here employed, a strain signifies a nematode culture derived from one host and not one derived from two or more. For the purposes of the experiments, it might have been more accurate to begin all cultures with one male and one virgin female obtained from one host. This was

attempted, but a long period often was required before the increase of the two initial parasites constituted a good growth and in addition a high mortality, probably due to intense inbreeding, occurred. Such a procedure was therefore impractical for present purposes. The method employed of placing about 1000 second-stage nemas on the surface of the medium yielded the most consistent results.

Cultures developed well at room temperatures, with an optimum of 22–28°C., and an attempt was made to keep within this range. High air temperatures, especially, during the summer months affected even the best cultures adversely.

EXPERIMENTS

An experiment, table 1, is presented to show the growth characteristics of Neoaplectana and the waning of development on prolonged contact with the standard medium, namely, dextrose-veal infusion agar with living yeast. These nematodes were obtained directly from parasitized grubs found in the field. It will be seen that no changes in the density of the growth occurred from the tenth to the twelfth day, and this illustrates why a 10-day culture was used in most cases for final appraisal. No change in the character of development occurred from the first through the seventh transplant. The data from the sixth through the twelfth transplant are represented. A waning of the culture became apparent at the eighth transplant and progressed to the twelfth, at which time all the nemas died.

In preliminary tests it was found that a powder prepared from desiccated, whole, third instar, Japanese beetle grubs, when sprinkled on the surface of waning cultures had a stimulating influence. After treatment healthy looking females were again observed, and these gave birth to the normal number of young, about fifteen. In a few cultures a heavier growth was obtained than was previously observed, indicating that more mature ova and young were formed. Later the same effect was produced, with a higher degree of constancy, by sprinkling a powder prepared from the desiccated, whole, bovine ovary on the waning cultures.[2] The reaction with ovarian substance is illustrated in table 2. The waning nematodes from the tenth transplant, table 1, were few in numbers but some from one plate were placed on nutrient dextrose agar with living yeast augmented by 0.02 gm. of ovarian substance. In 10 days excellent development was obtained. This culture was transplanted three times with the same constituents. At the end of this period the nemas were washed; 1000 placed

[2] Since then it has been found that a powder labelled "ovarian substance," prepared by Parke, Davis, and Company, Detroit, Michigan, is entirely satisfactory.

(fourth transplant) upon the surface of the standard medium, and 1000 upon the same medium with 0.02 gm. of ovarian substance. Under standard conditions the nemas did not grow and were completely lost on the second transplant. On the other hand, test no. 2, table 2, showed excellent results through the fifth transplant. This experiment (tables 1 and 2) showed that something was depleted in the tissues of the parasites and that

TABLE 1

Development of Each Transplant, from the Sixth, of a Nematode Strain from the Host on Dextrose-Veal Infusion Agar + Living Yeast

Transplant no. at 10-day intervals	Growth in days after transplantation					
	2	4	6	8	10	12
6	±*	+	++	+++	+++	+++
7	±	+	++	+++	+++	+++
8	±	+	+	+	++	++
9	±	+	+	+	++	++
10	±	+	+	+	+	+
11	±	±	±	±	±	±
12	0	0	0	0	0	0

* 0 = no growth ± = doubtful + = poor
++ = fair +++ = good ++++ = excellent

TABLE 2

Poor Growth from Tenth Transplant, Table 1, to Nutrient Dextrose Agar + Living Yeast + Ovarian Substance. In 10 Days Excellent Growth (++++) Transplanted to These Nutrients Three Times; Then Fourth Transplant as Below

Test no.	Nutrients	Transplantation no. after 10 days' growth				
		1	2	3	4	5
1	Dextrose + living yeast	±	0			
2	Dextrose + living yeast + ovarian substance	++++	++++	++++	++++	++++

three transplants (about six generations) with ovarian substance were not sufficient to replenish the tissues.

Test no. 2, table 2, was carried for eight transplants with ovarian substance (++++ growth). The ninth transplant (about eighteen generations), table 3, was tested as before. On the standard medium a slight effect was observed beginning on the fourth day, but the nematodes died on the tenth day. On the plate with ovarian substance the nematode growth became luxuriant on the tenth day. No. 2, table 3, was immediately tested

again at the tenth transplant and yielded the result given in table 4. In this case ten transplants with ovarian substance produced sufficient storage of the necessary growth material in the tissues so that the culture survived. Test no. 1, table 4, was transplanted to the dextrose agar, living yeast medium without ovarian substance every 10 days for five transplants, during which time it grew well, then waned again at the sixth, and died at the seventh transplant. Another strain also required ten transplants with ovarian substance and then survived on yeast alone for six transfers before dying. Still another strain required twelve treatments with ovarian substance before rejuvenation occurred. This strain subsequently, however,

TABLE 3

No. 2 (Table 2) Carried Eight Transplants with Ovarian Substance. Then Tested Again (Ninth Transplant)

Test no.	Nutrients	Growth in days after transplantation				
		2	4	8	10	15
1	Dextrose + living yeast	±	++	++	0	
2	Dextrose + living yeast + ovarian substance	±	++	+++	++++	++++

TABLE 4

Tenth Transplant of Nemas from No. 2, Table 3

Test no.	Nutrients	Growth in days after transplantation					
		2	4	6	8	10	12
1	Dextrose + living yeast	+	++	+++	++++	++++	++++
2	Dextrose + living yeast + ovarian substance	+	++	+++	++++	++++	++++

died during the third transplant on yeast alone. From the above observations it appears that separate strains of Neoaplectana vary in their response to the stimulating effect of ovarian substance.

The experiments were started with Neoaplectana obtained from parasitized grubs collected during June. The second-stage nematodes taken from Japanese beetle infested soil during the warm months yielded good cultures on yeast alone in early transfers, but nematodes obtained from samples of soil gathered in early spring (March 17, 18, or 20) could never be grown in this nutrient. However, the addition of ovarian substance, in the latter case, gave rise to good cultures. Apparently, the free-living stage of the nematode remaining inactive in the soil all winter became de-

pleted of growth material. In other words, this prolonged starvation period in nature accomplished the same result as continued artificial culture in the presence only of yeast cells. With the approach of warm weather, and after several natural passages through grubs, the second-stage forms recovered from soil had recuperated sufficiently to develop again on the standard medium.

Table 5 presents data on various materials tested on eight strains of Neoaplectana, the latter recovered directly from parasitized grubs after eight experimental grub passages to insure vigor. The second column shows the results obtained with dextrose-veal infusion agar, on which the natural flora, derived from the nemas, was permitted to develop. The

TABLE 5

Materials Tested on Eight Strains of Neoaplectana
(Observations Recorded on Tenth Day)

Test no.	Character of growth on materials tested								
	Natural flora	Heat-killed yeast	Living yeast						Ovarian substance + natural flora
			Alone	+ Grub substance	+ Ovarian substance			+ Orchic substance	
					Unaltered	Fractionally sterilized	Autoclaved		
1	0	0	++	++	++++	++++	+++	++	+++
2	+	0	+++	+++	++++	++++	+++	++++	+++
3	+	0	+++	++++	++++	+++	+	±	+++
4	0	+	+++	++++	++++	++++	+++	+++	+++
5	0	+	+++	++++	++++	+++	+++	+++	+++
6	+	+	+++	+++	++++				
7	0	0	+++	++	++++				
8	0	0	+++	+++	++++				

nemas either grew poorly or not at all. Collateral experience showed that many bacteria rapidly kill Neoaplectana. This was especially true of putrefactive species, but all bacteria encountered were more or less harmful. In the third column it is seen that heat-killed yeast was no more advantageous than bacteria. On the other hand, living yeast with a 24-hour start, as recommended, usually yielded good cultures. Living yeast with 0.02 gm. of grub substance usually produced good cultures, with three recorded as excellent. Living yeast with 0.02 gm. of bovine ovarian substance consistently produced excellent growths. Living yeast with the same amount of ovarian substance fractionally sterilized (Arnold sterilizer) gave a decreased effect in two cases, while living yeast with autoclaved ovarian sub-

stance gave no better results than living yeast alone. The use of bovine orchic powder, except in one case, showed no advantage. Ovarian substance without yeast, but with the natural flora of the nemas, stimulated growth, whereas natural flora alone did not. The data in the last column were comparable to those recorded under living yeast. These tests indicated that living yeast, grub substance, and ovarian substance each contained material necessary to the normal development of the nematodes. When living yeast and one of these substances were combined experimentally, the character of the growth was improved. This was especially true when yeast and ovarian substance were combined. It appeared that the factor which influenced the development of the ovaries in Neoaplectana was more highly concentrated in the bovine substance than in the powder prepared from grubs.

Table 6 records the number of times the eight strains were transplantable. The nematodes on their natural flora and on heat-killed yeast survived only a short time. On living yeast the shortest number of times a strain could be transplanted was three and the longest ten. On living yeast with grub substance the survival period of certain strains was prolonged, while on living yeast with ovarian substance this period was greatly increased. This value in the next column was again slightly influenced by fractional sterilization, and inactivation was again observed with the autoclaved material. The survival in the presence of orchic substance pointed to some stimulation as did the use of ovarian substance alone. From these survival values it followed that both yeast and ovarian substance were important for the nematodes while under cultivation. The highest transplant numbers recorded, like 16, 18, 19, 20, and 22, do not signify, as do the others, that the nemas died at that point. As a matter of fact, these cultures still showed no evidences of waning and might have survived indefinitely or at least for a much longer time. The highest values recorded merely signify that the experiments were terminated at that time. At any rate, a survival under artificial conditions of from 160 to 220 days is very good.

A variation in behavior was sometimes observed during the culture of various strains. A few strains grew better and survived longer than others under the same conditions. Some strains grew well enough initially, but did not survive many transplants no matter what substances were added. The best example encountered was strain 3, table 6. By glancing across the column transversely it will be seen that, no matter what materials were tried, the nematodes survived an extremely short time. This and other results merely point to the physiological variability of this particular organism.

The ability to keep certain strains of Neoaplectana from rapid extinction has proved of practical value in the rearing of this parasite in quantity for field distribution. The method outlined is now used as a matter of routine in the work conducted at the nematode laboratory at White Horse, New Jersey, jointly supervised by the State Department of Agriculture and the Federal Government.

The results with bovine ovarian substance suggested an effect produced either by a specific nutrient, by more than one nutrient, by a combination of these with a hormone, or by some particular hormone. For this reason certain sex hormones and other glandular preparations were tried alone and in conjunction with specific proteins. In each case the standard medium

TABLE 6

Same Nematode Strains on Same Materials as Shown in Table 5

Test no.	Times transplantable at 10-day intervals								
	Natural flora	Heat-killed yeast	Living yeast						Ovarian substance + natural flora
			Alone	+ Grub substance	+ Ovarian substance			+ Orchic substance	
					Unaltered	Fractionally sterilized	Autoclaved		
1	0	0	3	10	22	18	0	8	11
2	2	0	10	12	20	16	6	13	14
3	1	0	3	3	3	4	3	1	1
4	0	1	3	6	20	20	3	12	12
5	0	1	6	13	19	8	3	12	8
6	1	1	7	12	16				
7	0	0	7	9	12				
8	0	0	7	6	18				

with living yeast was considered basic, and the other materials were added to the surface either in 0.02 gm. amounts when powders, or in rat or other units when in the liquid form. During the course of 2 years, tests were performed on nematode cultures that had waned to see if they could be rejuvenated. Comparable tests were also performed on vigorous cultures direct from parasitized grubs. In these cases, the materials were added at each transplant to determine whether by so doing the nemas could be prevented from dying. Control cultures with living yeast with and without ovarian substance accompanied every test.

Powdered peptone, egg albumen, casein, and whole milk when used alone or in combination were inert. Other materials which proved inert singly

or in various combinations were Bacto-beef,[3] Bacto-liver,[4] Eli Lilly Co. liver substance No. 343, and a 0.5% sheep liver extract prepared by us. Bovine pituitary extract,[5] powdered whole bovine pituitary gland, and commercial antuitrin "S"[6] acted likewise.

Squibb's "Amniotin" and Parke, Davis, and Company's "Theelin" when used in concentrations of ½ to 25 rat units per culture showed no injurious nor any stimulating effect. The Schering Corporation's "Progynon-B" was also inert, insofar as the nematodes were concerned. Through the courtesy of Dr. Edwin Schwenk of the Schering Corporation the writer was presented with 10 mg. of Alpha-dihydro theelin crystals. These, he was informed, represented a hormone purified from ovaries. The material was not soluble in water and therefore had to be ground to a powder which was suspended in water before using. One to 10 rat units per culture were tried, but no reaction was obtained. The criticism might be offered that too high a concentration of this and other hormones was used in the tests. However, the rapid deleterious action of microorganisms on the materials used must be considered, and further, the ingestion of the materials by the worms probably adds another hazard to the operation.

After all this work, we were in the same position, namely, that stimulation of development in Neoaplectana by ovarian substance could not be attributed to any of its known components. This substance is fairly stable and will even cause development in nemas after storage in the refrigerator (5–7°C.) for at least 1 year.

SUMMARY

Prolonged culture of Neoaplectana glaseri, a parasitic nematode, on a standard medium produced sterile females. The sterility was prevented by host passage; by the use of desiccated material from the host; and by the use of desiccated, bovine, ovarian substance. After a number of generations in the host or in the presence of the desiccated materials, the parasites again usually developed on the standard medium for a large number of generations. The results indicated that some growth factor initially stored within the tissues was diluted with each generation until depletion occurred. This growth factor was restored to the parasite tissues by proper treatment over many generations. By testing various materials, it was found that,

[3,4] Prepared by Difco Laboratories, Detroit, Michigan; Bacto-beef from beef muscle, Bacto-liver from beef liver.

[5] Prepared by a colleague, Dr. Harry Greene.

[6] Parke, Davis, and Company, Detroit, Michigan.

under culture conditions, living yeast, host substance, and especially bovine, ovarian substance, were necessary to the prolonged normal development of the nematodes. When living yeast and one of these substances were combined, the character of the growth and survival was greatly improved. A variation in the growth and survival of certain strains was observed. The prevention of extinction of Neoaplectana cultures by the use of ovarian substance has proved valuable during the course of some economic work conducted jointly by the State of New Jersey and the Federal Government. Tests with certain sex hormones and other glandular preparations alone and in conjunction with various proteins failed to throw light on the nature of the stimulating factor or factors present in bovine ovarian substance.

LITERATURE CITED

Glaser, R. W. 1931 The cultivation of a nematode parasite of an insect. Science, vol. 73, pp. 614–615.

——— 1932 Studies on Neoaplectana glaseri, a nematode parasite of the Japanese beetle (Popillia japonica). Circular No. 211, Bureau of Plant Industry, State of New Jersey Department of Agriculture, Trenton, New Jersey. Reprinted in Studies from The Rockefeller Institute for Medical Research, 1932, vol. 83, pp. 521–550.

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EXSHEATHING AND STERILIZING INFECTIVE NEMATODE LARVAE

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To obtain easily and consistently the second ecdysis of strongyloid nematode larvae (thus releasing the third larval, i.e. the first parasitic stages) and secure them in quantity under sterile conditions, is a necessary aim of workers interested in the artificial culture of these forms. The second molt "skin" developed at the end of free-living existence remains as a resistant coat which in many species subserves a protective function by preventing dehydration and other deleterious effects of the environment. Normally this cuticle is shed at the time or after the parasite enters its host. While infective nematodes can be caused to migrate through ground glass and carborundum powder to tear the second molt cuticular covering from the third stage larva within, such methods are cumbersome and produce inconstant results. Moreover, when by these methods exsheathed first parasitic forms have been obtained, they are not sterile in the bacteriological sense and culture work with them is thus not possible.

Lapage was the first worker to develop an easy and practical method whereby the second ecdysis of large numbers of nematode larvae could be secured. This worker was able to obtain the species used under sterile conditions, so that the first parasitic stages could be put into any desired sterile medium, although he failed to get any further ecdysis or development in the many different media which he used.

In 1933 Lapage reported (1) that the infective stages of *Graphidium strigosum* and *Trichostrongylus retortaeformis*, nematodes of the rabbit, could be freed from their second stage sheaths by placing them in solutions containing hypochlorite, of which he found "Milton" to be best. In 1 part of Milton solution to 20 parts of distilled water, the larvae exsheathed in 20 minutes or less at laboratory temperatures. Stronger dilutions damaged or killed them. Lapage favored Milton because it remains stable and exsheathed larvae will live 3 or 4 days in the dilution of 1 to 20.

In 1935 Lapage (2) extended these important observations to *Haemonchus contortus*, *Ostertagia circumcincta*, and species of *Trichostrongylus*, nematodes parasitic in sheep. A number of different chemicals were tried successfully for the artificial production of ecdysis, but none were found as useful as Milton hypochlorite in respect to both exsheathing and sterilizing effects.

In another paper Lapage (3) more fully described the experimentally produced second ecdysis. The larva is passively ejected part of the way out through a hole at the anterior tip of the sheath and completes its emergence by its own movements. Lapage states: "Milton solution alters the chemical composition of the sheath with two results: (a) the permeability of the sheath is altered so that increased internal pressure results, by which the larva is propelled out of the hole at the anterior tip, and (b) the sheath is rendered soluble in the NaOH in the Milton solution so that it is subsequently dissolved. The larva is expelled through a hole at the anterior tip before the whole sheath is dissolved because the sheath gives way here first to the combined effects of the increased internal pressure and the solvent action of the NaOH, this being its weakest point, where the remains of the mouth of the second larva are." He concludes by stating: "Further investigations of the second ecdyses by biophysical methods may bring all the ecdyses of parasitic nematodes under experimental control and so make possible the artificial cultivation of the adult parasites."

EXPERIMENTAL

In our attempts to cultivate the parasitic stages of certain sheep nematodes (4) it became imperative to obtain quantities of exsheathed and sterile third stage forms. At first we were unable to obtain Milton solution, so decided to use a similar, easily obtained product known as "Labarraque's solution" containing not less than 2.6 per cent of sodium hypochlorite in contradistinction to 1.01 per cent in Milton. Like the latter preparation, Labarraque's solution is stated to be a deodorizer, preservative, insecticide, and general antiseptic. It is said not to be so stable as Milton solution and should be stored in well-stoppered bottles, in a cool place, protected from light. A 1 to 20 dilution of Labarraque's solution readily caused the exsheathing of the infective stages of *Haemonchus contortus*, *Ostertagia circumcincta*, and *Trichostrongylus axei* of sheep; *Obeliscoides cuniculi* of rabbits; and *Nippostrongylus muris* of the rat. Presumably it will act similarly on strongyloid forms generally.

The details of the process of exsheathing in Labarraque's solution com-

pare in general with those described by Lapage for Milton solution. However, we do not believe that the movements of the larva are essential in completing its emergence from the sheath. When the mature free-living larvae of *Haemonchus contortus* are rapidly killed in hot water and then placed in the Labarraque dilution of 1 to 20, the dead, rigid forms are ejected from their sheaths as readily as the living larvae. We therefore think that the solvent action of Labarraque on the sheath and the increased intrasheath pressure produced are probably the main factors concerned. The fact

FIG. 1. Infective *Haemonchus contortus* larvae in their end-2nd stage sheaths. Heat-killed. ×90. (Photograph by J. A. Carlile.)

that the living larvae lash violently when the anterior tip gives way, no doubt assists the ejection materially, but the movements are not essential. The chief factors appear to be physico-chemical.

In Fig. 1 are illustrated the infective larvae of *H. contortus*, and in Fig. 2 the exsheathed forms after Labarraque treatment. The wrinkled and somewhat telescoped appearance of the cast sheaths is characteristic. There is subsequent solution and disappearance of the sheaths during exposure to the hypochlorite.

While exsheathing was thus readily accomplished, in our hands and with our methods, it has not been so simple to surely *sterilize* the larvae of *Hae-*

monchus contortus, *Ostertagia circumcincta*, and *Trichostrongylus axei*. Not infrequently bacteriological tests with routine laboratory media even after a week's incubation seemed to show that the larvae were sterile. However, when such larvae were placed in our enriched and partially anaerobic media used for nematode culture, bacteria or fungi often appeared, sometimes even after an incubation of two or three weeks. Contaminated

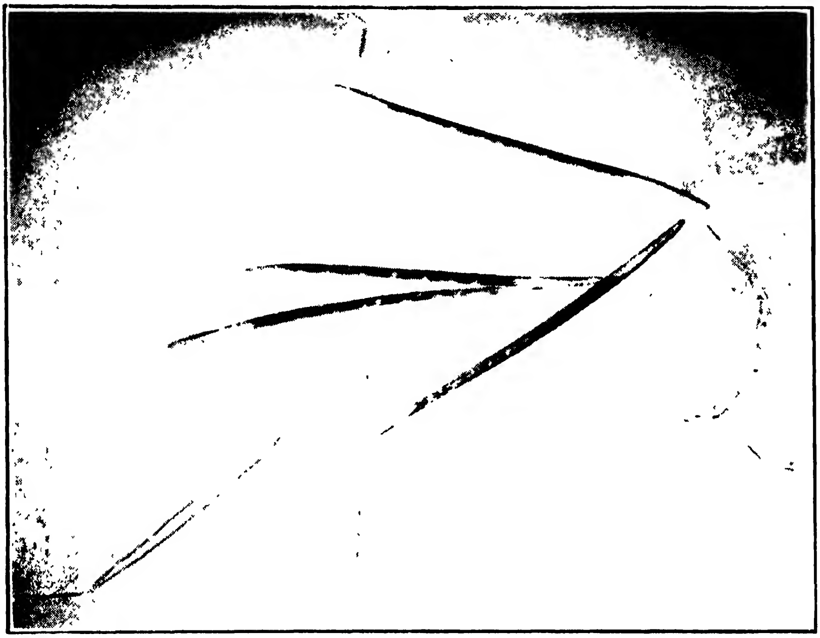

FIG. 2. Infective *Haemonchus contortus* larvae (heat-killed) which have been exsheathed in Labarraque's solution. The attenuated tail of the 2nd stage larvae shows in the cast sheaths, in contrast to the more stubby posterior tip of the free 3rd stage forms. ×90. (Photograph by J. A. Carlile.)

culture tubes are of no value, since parasitic stages of the nematodes here discussed only showed development in the absence of living microörganisms.

More effective sterilization resulted, however, when we lengthened the washing and sedimenting stages to be described. This lengthened period evidently allowed more of the microörganisms contained within the larvae themselves to be expelled, presumably by normal peristalsis. Once free, the bacteria were then either lost during the washing procedures, or were rendered available to chemical attack by the hypochlorite.

The following method, slightly modified from time to time, has yielded sterile larvae with very few exceptions during the past two years. The

mature free-living infective larvae in feces are isolated in a "Baermann" funnel after which they are settled four times in 15 cc of sterile water. During this procedure a considerable amount of clumping may occur. The larger clumps as they settle together with coarse débris must be removed with a fine pipette and discarded. The nematode suspension should never be too dense; otherwise the occurrence of clumping adds to the danger of carrying along an excessive amount of contamination. Later on, should the nematode suspension be of the proper density for future work, and should a small degree of aggregation still occur, the clumps must be broken by sucking them in and out of a fine pipette.

The nemas are now permitted to settle in a sterile, long, glass tube held in a vertical position, containing about 15 cc of sterile water.* The nematodes gradually settle during the course of approximately one hour into the tapering end of the tube. The end of the tube is then inserted into 95 per cent alcohol for a few minutes. At the same time a file is inserted into the alcohol and flamed. The long tube is cut at the constriction with the sterilized file and the nemas transferred, with a small amount of the water, from the tapering end to a small sterile test tube (agglutination tube) by means of a sterile, finely drawn pipette. When the nemas have settled, the small amount of water is drawn off and about 5 cc of Labarraque's solution (or Milton's), diluted in the proportion of 1 part to 20 parts of water, introduced. After the nemas have again settled, the solution is drawn off and 5 cc of a fresh supply of Labarraque's solution added. This second addition of Labarraque is made because the first lot is still diluted beyond the 1 to 20 dilution by some original water associated with the larvae. In this second lot of Labarraque the nemas remain for ½ hour. During this time they become exsheathed and superficially sterilized.

At the end of this period the Labarraque is drawn off and the larvae are again settled in one of the long tubes. After settling and after the tube and file have been treated as previously described, the larvae are removed aseptically from the tapering tip and transferred to a tube containing 10 cc of sterile water. Here the parasites remain for 48 hours with two changes

* This tube, first used in 1930 (5) for purifying protozoa, has proved very helpful in work with nematodes. The tube is about 14 inches long with a ½ inch bore. The upper end is plugged with cotton and the lower end has a coarsely tapering point, 2 to 2½ inches long, having a slight constriction where the taper starts. The end of the taper is sealed in a flame after the tube is filled with sterile water.

Agitating the nema suspension by rotating the tube vigorously at intervals between the palms of the hands facilitates the settling.

of sterile water during this time. This prolonged sojourn in water permits the larvae to digest and excrete viable bacteria within their alimentary tracts. Cultures taken from larvae at the end of the first day, i.e., up to the 48 hours' sojourn in water, often prove sterile when routine media are used. However, much time, energy, and material are saved if the procedure is properly concluded. As stated previously, special enriched media are used for our nematode culture work and these media often in time reveal the growth of microörganisms not shown by the routine methods. However, sterile cultures obtained at the end of this cleansing period signify that the nemas have been freed of much of their flora.

At the end of the 48-hour period the larvae are again settled in sterile water in one of the long tubes. They are then removed and placed in a 1 to 20 dilution of Labarraque freshly prepared. This is removed after the larvae have settled and fresh Labarraque added. Here the nemas are subjected to a further sterilization for ½ hour. The Labarraque is then drawn off and the larvae are again settled in a long tube. After removal from the long tube, in the customary manner, they are transferred three times to three separate lots (5 cc) of sterile Ringer's or Tyrode's solution of one-fourth concentration. From the last tube the nemas may be transferred to any desired medium with the assurance that the culture will remain sterile.

The above procedure is now standard in our laboratory and has been successfully performed as a routine by a laboratory technician for over a year. Other duties can be performed while the sterilization work progresses, but one must be careful not to undertake the steps mechanically; otherwise, mistakes will occur.

Table 1 represents results of work that was done primarily with Labarraque's solution to determine its stability at various ages, at refrigerator temperatures (5–7° C), and at room temperatures (22–30° C). In so far as our work was concerned, the type of stability that interested us was not absolute chemical stability, but power to exsheath, to dissolve the cast sheaths, to sterilize, and to permit the nemas to develop subsequently in media. Later, we were able to procure and compare two lots of Milton's solution with Labarraque, and two tests with Carrel-Dakin's solution were also included.

Many experiments were performed and from these a representative number were chosen for Table 1. It will be seen that no correlation exists between the age of Labarraque's solution and the speed of exsheathing or the dissolution of the cast sheaths. The solutions were stored in dark bottles and, except in the marked instances, held in the refrigerator. In

four tests samples of Labarraque held at room temperatures for from 59 to 64 days were used. One such test is shown in the table and the results are identical with those given above. The data with recent and old Milton are similar to one another and to Labarraque. With Carrel-Dakin's solution the time seems to be considerably extended.

TABLE 1

Summary of Tests on the Effects of Hypochlorite Solutions on Exsheathing, Sterility, and Development into the 4th Stage of Haemonchus contortus Larvae

Test No.	Solution	Age of solution in days	Exsheathing in minutes		Dissolution of cast sheaths in minutes		Sterility	Parasitic development in media
			Start	End	Start	End		
1	Labarraque	1	5	8	3	10	+	+
2	"	7	3	5	10	15	+	+
3	"	16	5	10	10	20	+	+
4	"	23	5	8	3	8		
5	"	29	5	10	5	10		
6	"	37	5	15	5	18	+	+
7	"	48	17	20	15	22		
8	"	51	13	20	5	10		
9	"	63	5	10	5	8		
10	"	72	10	14	5	15	+	0
11	"	93	5	8	3	5		
12	"	98	5	8	3	5		
13	"	190	5	8	3	5	+	+
14*	"	59	3	5	5	10	+	+
15	Milton	1	3	5	5	9	+	+
16	"	365	5	8	3	5	+	+
17	Carrel-Dakin	3	10	15	15	35	0	0
18	"	5	10	15	15	30	+	0

* All solutions stored in refrigerator at 5–7° C, except No. 14 held at room temperature, 22–30° C.

In most of our culture work Labarraque's solution one week old was used and no attempt was made to correlate age with power to exsheath. The table supplies these lacking data. A representative group in this series was carried further, i.e., as regards sterility and ability of the nematodes to initiate development when placed in appropriate media. Sterility of the nemas and their environment was checked initially and again at the end of each experiment. By development in media is meant some degree of growth in the fourth larval (second parasitic) stage (4).

From the data it is seen that Labarraque, stored at 5–7° C, is effective as regards sterility and in permitting development after storage ranging from

one day to 190 days. Labarraque held at room temperature for 59 days yielded sterile larvae which developed normally. The same thing obtains for Milton solution held at 5–7° C. In one test with Carrel-Dakin's solution the nematodes were not sterilized and naturally no development occurred. In another test (No. 18) sterility was obtained but no development occurred. However, this test had no control with Labarraque sterilized larvae and the same combination of nutrients, so that the results may not be certain as regards inability to grow after Carrel-Dakin treatment.

SUMMARY

The second ecdysis of certain nematode larvae can be readily produced by placing them in Labarraque's solution at a dilution of 1 to 20 with water. Nematodes so treated include *Haemonchus contortus*, *Ostertagia circumcincta*, *Trichostrongylus axei*, *Obeliscoides cuniculi*, and *Nippostrongylus muris*. The details of the process of exsheathing in Labarraque's solution compare in general with those described by Lapage for Milton's solution. We feel, however, that physico-chemical factors are the most important. A method is described, incorporating the use of Labarraque's or Milton solution, whereby sterile, uninjured nematodes are assured. Labarraque's solution, especially if stored in the refrigerator, remains stable for a long time, at least as regards its power to exsheath and sterilize. Separate lots at different ages have not proved toxic when used at the proper dilution, as evidenced by culture results. In this sense Milton solution is stable also. The results with Carrel-Dakin's solution are uncertain.

BIBLIOGRAPHY

1. Lapage, G. 1933 Cultivation of parasitic nematodes. Nature **131**: 583.
2. ——— 1935 The second ecdysis of the infective larvae of certain Trichostrongylidae in solutions of sodium sulphide and of organic compounds containing sulphur. J. Helm. **13**: 103.
3. ——— 1935 The second ecdysis of infective nematode larvae. Parasitology **27**: 186.
4. Glaser, R. W. and Stoll, N. R. 1938 Development under sterile conditions of the sheep stomach worm *Haemonchus contortus* (Nematoda). Science **87**: 259.
5. Glaser, R. W. and Coria, N. A. 1930 Methods for the pure culture of certain protozoa. J. Exper. Med. **51**: 787.

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SOME PROPERTIES OF THE ENCEPHALOPATHIC AGENT IN PRIMATE BONE MARROW (THE GORDON AGENT)

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PLATE 29

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The so called "Gordon agent" (1) is present in the lymph nodes of Hodgkin's disease, as well as in other normal and pathologic tissues of both man and animals. Intracerebral inoculation of this agent into susceptible hosts produces clinical disease and distinctive pathologic damage, fully considered in a previous paper (2), and characterized chiefly by selective necrosis of Purkinje cells.

The nature of this pathogenic agent has received considerable attention in the literature. That it is not restricted to Hodgkin's disease tissues was soon apparent. Gordon (3) obtained the characteristic clinical reaction from injection into rabbits of human pus. Friedemann and Elkeles (4) and Friedemann (5) demonstrated the agent in normal human bone marrow, spleen, and the leucocytic cream of normal human blood, as well as from marrow from "acute leukemia." Although Van Rooyen (6) could not demonstrate the importance of eosinophilia, Turner, Jackson, and Parker (7), and McNaught (8) showed convincingly that the potency of the test material derived from human beings was roughly proportional to the number of eosinophils in the inoculum. That the agent is not restricted to the primate eosinophil was proved by Edward (9), who produced the characteristic result with mouse spleen and with chicken spleen and marrow. The potency of mouse spleen has been confirmed by the present author. The relationship of mouse and chicken eosinophils to those of primates is not unequivocal. What cell type in the lower animals gives rise to the agent responsible for the phenomenon cannot at present be decided. It has been shown by MacKenzie and Van Rooyen (10) that the pathogenic agent is not identical with known proteolytic enzymes derived from leucocytes.

A few of the elementary properties of the Gordon agent have been described in the literature. It is inactivated by a temperature of 75-80°C. acting for half an hour (1, 11). Uhlenhuth and Wurm (11) found activity retained at a pH of 3.45, although Van Rooyen earlier observed inactivation below pH 6.8. The agent is readily adsorbed by kaolin (11) and kieselguhr (12). It is resistant to extreme cold (12) and desiccation (1) and may be kept desiccated or in glycerin for long periods of time. In addition the agent resists x-ray (12), weak phenol, and formalin, as well as ether and chloroform (1, 11). It is destroyed by antiformin acting for 30, but not for 15 minutes. Successful filtration can be accomplished with difficulty only if the filter is properly lined and atten-

tion paid to pH (12). Edward (13) attempted ultracentrifugation, and could find no evidence of sedimentation. Indeed, after high speed centrifugation the supernatant fluid was sometimes more active than control uncentrifuged material. He suggested either a lipoid nature of the agent or adsorption to lipoid particles.

All authors are now agreed that the agent is not transmissible in series from one affected animal to normal animals. The active agent, injected intracerebrally into a susceptible host, exerts its effect but cannot be recovered from the brain. The suggestion has been advanced in a previous paper (2) that the action may be an interference with cell respiration.

It has been shown previously (14) that the action of the agent is exerted on heterologous species only. Monkey bone marrow, potent against guinea pigs and rabbits, is harmless against monkeys. Recently Edward (9) has shown that although chickens are susceptible to the active agent, and although chicken spleen contains the agent, injection of chicken tissue into chicks is without pathologic effect.

No unequivocal evidence of immunological reactions has been brought forward in regard to the Gordon agent. This aspect has been most recently considered by Edward (13).

The present communication reports further studies on the nature of the encephalopathic agent in monkey bone marrow.

Material and Methods

In the present experiments the active agent under investigation was derived exclusively from monkey bone marrow. Most of the monkeys were supplied by friends and colleagues, and had been used in virus and immunological investigations. Several others were purchased from dealers expressly for the present study. No difference could be detected between the used and unused animals. Monkeys suffering from tuberculosis, and those dead of poliomyelitis, furnished extracts as potent as normal controls. There was considerable variation in the potency of marrow of different animals, but this variation could not be correlated with any factor of previous exposure to experimental conditions. Unused monkeys varied similarly.

Preparation of Extract.—The bone marrow was prepared and treated in the following manner. The long bones of the extremities were removed, the epiphyses sawed off, and the marrow was pushed out with an applicator. 4 to 6 gm. of marrow were obtained from a single animal. The bone marrow was then placed in about 15 volumes of acetone where it remained several hours to several days. The acetone was filtered off, and the residue washed with absolute alcohol, followed by ether, and then allowed to dry on the filter paper for several hours. The dried marrow weighed approximately one-fifth of the initial amount. A small proportion of this difference represents actual loss of solid material, but most of it is accounted for by removal of lipoids and water. The dried extract was mixed with about 15 volumes of 50 per cent glycerin. An exact measure was of no significance, because of normal variations in potency.

The material was kept in glycerin in a refrigerator. To prepare extract for injection, the glycerin was cleared of the solid material first by filtering through cheesecloth, and then by centrifugation. An aliquot of the glycerin was mixed with about 5 volumes of a mixture containing 3 parts of absolute alcohol and 1 part of ether. The copious precipitate was collected by centrifugation and, after the absolute alcohol-ether mixture

had been drained off, was suspended in buffered saline of a pH of 7.4. The amount of saline added depended on the potency of the material determined by preliminary tests. As a rule of thumb, the precipitate from 1 ml. of glycerin was taken up in 1 ml. of saline, but the proportions were readily varied if the bone marrow was especially strong or especially weak.

Saline suspensions of bone marrow, without any previous extraction, are potent. But use of a saline suspension is complicated by fatty substances in the inoculum and does not lend itself to storage of the material. Consequently in all experiments herein reported the inoculum was prepared according to the method given above.

Quantitative Estimations.—The active agent can be detected only by biological test. The quantity of precipitate thrown down from the glycerin by the action of the absolute alcohol-ether mixture furnishes no indication of the strength of the final preparation. For measuring the activity of a given sample, intracerebral inoculation into guinea pigs was employed. The animals averaged between 250 and 300 gm. in weight, a few being lighter, a few heavier. The dosage was 0.20 cc.

Injection of potent material resulted in death, or in symptoms with partial or complete recovery. As pointed out in a previous paper (2) the effect of the agent may be detected histologically as well as by the clinical syndrome already described. The histological picture, in reference to the damage to the Purkinje cells of the cerebellum, was absolutely constant and invariable, and much more reliable than clinical observation of the animal. A combination of both clinical and pathological observation, together with dilutions of the inoculum, furnished a rough quantitative measure for the procedures reported below.

The clinical severity was graded from 0 to ++++. Zero signified that at the termination of the experiment the guinea pig was free of symptoms and indistinguishable from normal. A mild degree of clinical disturbance may have been present but had totally regressed. In such instances histological examination revealed characteristic damage. A clinical designation of ++++ meant that the animal was unable to rise. A mark of +++ meant the animal was prostrated at times but could still regain its feet on occasion. Marks of + or ++ indicated ataxia and incoordination of greater or less degree, but without prostration. When death occurred before the end of the experiment it was so indicated.

For histological grading, the cerebellums of the experimental guinea pigs were sectioned in the sagittal plane, and midsagittal sections stained with toluidine blue. As already described, the essential pathological process—the destruction of the Purkinje cells—first takes place around the periphery. A mark + indicated that a portion only of the peripheral Purkinje cells was destroyed, but the damage was definite, characteristic, and unmistakable; ++, that the destruction extended around almost the entire periphery; +++, that the more deeply placed folia were markedly affected; and ++++, that the destructive process was maximal in intensity over the entire section (Fig. 2).

In case of a difference between clinical and pathological observation, the latter must be the final criterion. Clinical disturbance, when mild, may be non-specific. In such instances a histologically normal cerebellum required a dismissal of slight symptoms as unrelated to the disease picture. On the other hand, symptoms might be transient and questionable, yet if unmistakable pathologic disturbance was present the animal was judged affected by the disease process.

OBSERVATIONS

Susceptible Hosts.—Although all the experimental work detailed below was performed with guinea pigs, other animals are susceptible. Rabbits were first used by Gordon and by other investigators. Recently Edward (9) has shown that chickens may be affected by the agent derived from human bone marrow. This has been confirmed. Young chicks were found to respond to monkey bone marrow extracts in a way generally similar to guinea pigs, with identical pathologic changes in the cerebellum and the blood vessels at the base of the brain. However, chicks reacted much less constantly and less sensitively than guinea pigs. Clinical symptoms were also much more difficult to determine in the fowl. For routine use guinea pigs were superior to either rabbits or chicks.

Effective Routes of Inoculation.—The active agent is effective when injected directly into the brain substance or into the cisterna magna without injury to brain tissue. Subcutaneous and intraperitoneal inoculations were without pathologic effect, even if the brain was simultaneously traumatized. Injection into the eyeball, whereby the agent is in contact with nerve cells although not with cerebrospinal fluid, did not produce the disease picture.

Stability of the Agent.—Although systematic experiments were not performed, certain incidental observations may be recorded. The monkey bone marrow preserved in glycerin in the refrigerator remained potent for at least 7 months, in excellent agreement with the data on Hodgkin's disease tissue. The final buffered saline suspension of material precipitated from glycerin remained active when kept at room temperature for 17 days. This corrects a previous observation (14) that the final extract became inert on standing 24 to 48 hours. Bone marrow kept in acetone in the refrigerator for 4 months was found to be fully active when the remaining stages of preparation of the extract were then performed.

Solubility of the Agent at pH 7.4.—When the precipitate from the glycerin is mixed with saline it is never completely dissolved. In many experiments use of the entire resulting suspension was entirely satisfactory. But frequently a solution of the agent free from particulate matter was essential. It was necessary, therefore, to determine the relations between the amount of active agent in solution and in the whole suspension. As will be shown subsequently the amount of agent in solution depends on the hydrogen ion concentration. For routine use, physiological salt solution buffered at pH 7.4 with phosphate buffers was employed.

A suspension of bone marrow extract was made up in buffered saline and allowed to stand overnight at room temperature. After thorough agitation a portion was removed

to serve as a sample of whole suspension. The remainder was centrifuged in a horizontal centrifuge for 1 hour at a speed of approximately 2000 R.P.M. The supernatant fluid was then drawn off. The whole suspension and the supernatant were diluted and injected into guinea pigs which were allowed to live 6 days.

The results are shown in Table I. The whole suspension is very slightly more active than the supernatant fluid alone, but the difference is not great. The active agent is highly soluble at pH 7.4, and such a solution contains practically all of the agent present in any given sample.

TABLE I

Comparison of Whole Suspension and Supernatant Fluid (pH 7.4) after Horizontal Centrifugation

Inoculum	Dilution	Guinea pig No.	Result 6 days after inoculation	
			Clinical	Pathological
Whole suspension	1:12	1	±	++
		2	+	++
	1:4	3	++	++
		4	++	++
	1:1	5	+++	+++
		6	++++	++++
Supernatant after centrifugation	1:12	7	±	±
		8	±	+
	1:4	9	+	++
		10	++	++
	1:1	11	++++	+++
		12	+++	+++

Heat Inactivation.—The thermal inactivation point for the active agent contained in Hodgkin's disease tissue has been determined by previous investigators. It was deemed of interest to establish data in regard to monkey bone marrow.

A clear solution of bone marrow extract at pH 7.4 was obtained by centrifugation. Portions were heated in water baths at temperatures shown in Table II, and then inoculated into guinea pigs. In cases where precipitate formed as a result of the heat treatment, the suspended precipitate was included in the inoculum.

The results of two such experiments are shown in Table II. Although the solutions were initially clear, heat of 70°C. or over resulted in a precipitate of coagulated protein, minimal but yet definite at 70°, and cor-

respondingly more at higher temperatures. The use of temperatures of 60° or 65° did not result in precipitate.

TABLE II

Inactivation of Active Principle by Heat

Experiment	Inoculum heated		Guinea pig No.	Results at end of experiment	
	Time	Temperature		Clinical	Pathological
		°C.			
1	15 min.	80	1	± to +	±
			2	0	0
		75	3	±	0
			4	±	0
		70	5	0	+
			6	±	+
		65	7	Died, 9 days	+++
			8	±	±
		60	9	++++	++++
			10	Died, 9 days	+++
	Control, unheated		11	Died, 6 days	+++
			12	Died, 6 days	++++
2	20 min.	100	13	±	0
			14	0 to ±	0
		90	15	0	0
			16	0	0
		80	17	± to +	0
			18	±	0
		70	19	0	0
			20	±	+
	Control, unheated		21	Died, 8 days	+++
			22	Died, 8 days	++++

Experiment 1. Surviving guinea pigs sacrificed after 12 days.
Experiment 2. Surviving guinea pigs sacrificed after 10 days.

At 60° there is apparently no diminution in potency, but potency is reduced at 65°, still further reduced at 70°, and absent above that point. Guinea pig 1 in Table II showed very questionable changes. Repetition,

as shown in the table, indicated the lack of significance of this questionable instance. The results, indicating retention of potency at 60°, with diminution in the next 10 degree temperature range, and inertness above 70°, are in fairly close agreement with the results of Gordon (1) and Uhlenhuth and Wurm (11) with Hodgkin's disease tissue. The inactivation point is slightly lower with the monkey bone marrow.

A further experiment was performed in which quantitative comparisons were made between material heated at 60° for an hour and unheated controls. With dilutions of 1:10, 1:4, and 1:1 (undiluted) the heated samples proved to be slightly less active than unheated controls, but the difference was not great.

Solubility of Agent at Various Hydrogen Ion Concentrations.—In all the experiments described in this paper, the agent was precipitated from glycerin by the action of absolute alcohol and ether. This precipitate, when injected as a whole suspension, was active over a wide range of hydrogen ion concentrations. Preliminary experiments showed that when this precipitate was mixed with saline at pH 2.8, 3.2, and 4.7, allowed to stand at these acidities overnight, neutralized, and then injected as a whole suspension into guinea pigs, the animals contracted the typical disease picture, both clinically and pathologically.

It is thus apparent that the agent is not inactivated by relatively high hydrogen ion concentrations. The question remains, however, regarding the solubility of the agent at these different concentrations. The active principle is almost entirely soluble at pH 7.4 (Table I). It remained to determine the relative solubilities at lower pH.

To each of 5 centrifuge tubes containing 10 cc. of absolute alcohol-ether mixture, 2 cc. of agent-containing glycerin were added. The resulting precipitates were centrifuged down, and the supernatant liquid was drained off. Buffer solutions were added to each tube to produce a final pH of 2.0, 3.0, 4.2, 5.0, and 6.8. The first two buffers were HCl-glycine, the second two, acetic acid-acetate, and the last, phosphate. The final result was determined by indicators, and is approximate only. The tubes were allowed to stand overnight, about 21 hours, and then centrifuged at about 2500 R.P.M. for an hour. Perfectly clear supernatant fluids resulted. From each sample 1 cc. was removed into small test tubes, a drop of phenol red added, and the pH adjusted to approximately 7.2. Since different amounts of alkali were added to different tubes, all were brought to a constant volume by the addition of appropriate amounts of distilled water. From each tube one portion was saved for use undiluted (1:1); a second portion was diluted 1:6. Intracerebral injections into guinea pigs were then performed. Some denaturation of protein occurred in the tubes of pH 2.0, and much less at pH 3.0. This was shown by the formation of a precipitate as the acid solution was neutralized. In such instances the entire resulting suspension was injected.

In Table III the results of this experiment are set forth. The results are fairly clean cut. At pH 6.8 the active agent shows a high degree of solubility, while at pH 2.0 and 3.0 there is also high solubility. At intermediate

TABLE III

Solubility of Agent at Different Hydrogen Ion Concentrations

pH	Dilution	Guinea pig No.	Results, 6 days after inoculation	
			Clinical	Pathological
2.0	1:6	1	+	++
		2	+	++
	1:1	3	Died, 3 days	+++*
		4	+++	+++
3.0	1:6	5	+	+
		6	+	++
	1:1	7	+++	++
		8	+++	+++
4.2	1:6	9	0	0
		10	0	0
	1:1	11	±	0
		12	0	0
5.0	1:6	13	0	0
		14	0	0
	1:1	15	+	++
		16	0	0
6.8	1:6	17	++	++
		18	0	+
	1:1	19	++	+++
		20	+++	++++

All inocula adjusted to pH 7.2 and made up to equal volume before dilution.
* Pathological evaluation difficult because of postmortem changes.

hydrogen ion concentrations the solubility is negligible, absent at 4.2, and very slight at 5.0. These results indicate a point of minimal solubility of the agent at approximately pH 4.2.

Digestion by Pepsin.—Evidence on the possible protein nature of the agent might be furnished if the activity were destroyed by pepsin. Of the various protein-splitting enzymes pepsin is well suited for this test. The

low pH required for its activity does not affect the agent under investigation. Furthermore, since the biological test requires intracerebral inoculation, it is necessary that the excess of enzyme be completely inactivated before injection. With pepsin this is readily accomplished by simple neutralization of the inoculum.

A suspension of active bone marrow extract was made, using 0.1 normal hydrochloric acid as the diluting fluid. One portion was saved for control. To the remainder, an excess of pepsin was added (1 mg. of purified crystalline pepsin, kindly furnished by Dr. Roger M. Herriott). Both portions were allowed to stand at room temperature overnight. In the morning both samples were neutralized with sodium hydroxide and injected into guinea pigs, 5 animals being used for each test.

TABLE IV

Effect of Pepsin on Active Agent

Inoculum	Guinea pig No.	Result after 28 days	
		Clinical	Pathological
Extract in 0.1 N HCl plus pepsin	1	0	0
	2	0	0
	3	±	0
	4	0	0
	5	0	0
Extract in 0.1 N HCl. No pepsin (Control)	6	Died, 8 days	NE
	7	+	+++
	8	Died, 11 days	NE
	9	Died, 9 days	NE
	10	++++	++++

Inocula neutralized before injection.
NE = not examined.

The data on this experiment are presented in Table IV. This experiment was allowed to run 28 days. Of the control group 3 animals died after typical clinical signs. The remaining 2 survived with symptoms, but showed marked characteristic lesions at autopsy. Guinea pig 7, at the termination of the experiment, showed only slight residual symptoms. Much more severe symptoms, present earlier, had partially regressed in the long period following inoculation. The pathological injury, however, never regresses. Of the group of animals receiving the pepsin-treated extract, all survived with no clinical disturbance except for one questionable instance (No. 3) and with no histological evidence of injury. As has been repeatedly emphasized, questionable and very slight symptoms are of no significance unless confirmed by pathological examination.

To rule out the possibility that this inactivation by pepsin was simple instantaneous inhibition rather than true digestion (15), the experiment was repeated with the following variations. A clear solution of bone marrow extract in 0.1 N HCl was treated with pepsin, and samples were withdrawn at intervals for neutralization. After neutralization the samples were all injected at the same time at the expiration of the last interval.

As seen in Table V, the first sample, neutralized after a lapse of about 10 seconds, remained active and is identical with the control, while subsequent samples were completely inactivated. The conclusion may be drawn that the inactivation represents a true digestion.

TABLE V

Time Relations of Pepsin Inactivation

Pepsin acting for	Guinea pig No.	Results after 7 days	
		Clinical	Pathological
10 sec.	1	++++	+++
	2	Died, 6 days	+++
1½ hrs.	3	+	0
	4	*	0
12 hrs.	5	0	0
	6	0	0
Control (12 hrs.)	7	Died, 6 days	+++

* Died on 7th day of intercurrent disease.

Ultracentrifugation.—The only experiments recorded in the literature concerning the possible sedimentation of the active agent by high speed centrifugation are those of Edward (13). He used human bone marrow or lymphadenomatous glands, suspended in saline or 5 per cent glycerin, and cleared of particulate matter by low speed centrifugation and filtration. Portions of this material were centrifuged at 40,000 R.P.M., and the supernatant was compared with uncentrifuged control solutions to test for possible diminution of activity. The titer of the supernatant after ultracentrifugation was generally slightly higher than the uncentrifuged control. Edward suggests that either an essential lipoid nature or an adsorption to lipoids may account for this result.

Preliminary experiments with monkey bone marrow, prepared according to the directions given in Methods, did not confirm these findings. The following description shows a more detailed experiment.

An extract was prepared as usual from the glycerin mother-liquor by precipitation with absolute alcohol-ether. The precipitate was taken up in saline at a pH of 7.4 and allowed to stand overnight. The next morning the suspension was spun in a horizontal centrifuge for 1 hour at about 2700 R.P.M. and the perfectly clear amber supernatant fluid removed. 5 cc. of this was placed in an air-driven centrifuge, which was run at 33,000 R.P.M. for 1½ hours. A slight red pellet about the size of the heads of 2 common pins was formed. The supernatant two-thirds was carefully removed and subjected to a second run of 1½ hours at 33,000 R.P.M., and the uppermost third after the second run was removed for inoculation. At the same time the bottom third remaining after the first run was utilized for comparison. The pellet, presumably hemoglobin or a hemoglobin complex, was redissolved before use. The 2 samples were diluted and injected into guinea pigs.

TABLE VI

Effect of Ultracentrifugation on Solution of Active Agent

Inoculum	Dilution	Guinea pig No.	Result after 6 days	
			Clinical	Pathological
Uppermost third after two centrifugations	1:12	1	0	±
		2	0	0
	1:4	3	+	+
		4	++	++
	1:1	5	++++	+++
		6	+++	+++
Bottom third of first centrifugation	1:12	7	+	++
		8	+	++
	1:4	9	++	++++
		10	++	+++
	1:1	11	++++	++++
		12	++++	++++

In this experiment out of a given sample the uppermost third after two high speed centrifugations was directly compared with the bottom third after the first centrifugation. The results, given in Table VI, show that the uppermost third is slightly but definitely less active than the lowermost third. This indicates a slight degree of sedimentation of the agent, but totally insufficient for purposes of purification.

The differences between these results and those reported by Edward are probably to be explained by the different methods of preparation of the material. In the present case, the preliminary treatment with acetone,

with the use of alcohol-insoluble precipitate to prepare the final solution, got rid of most of the lipoids which apparently complicated the experiments of Edward.

Dialysis.—For possible purification of the agent it was necessary to determine whether it would be retained by cellophane or collodion membranes. In one experiment a solution of bone marrow extract was dialyzed for 22 hours in a rocking dialyzer against running distilled water through a cellophane membrane. Injection of the dialyzed material into 3 guinea pigs resulted in death, with characteristic symptoms, after 8, 9, and 10 days, respectively. At the same time control undialyzed solution killed 3 guinea pigs in 8, 8, and 19 days. Histologic examination was not done on these animals. The results show no evidence of loss of activity through dialysis.

A more detailed experiment was carried out as follows:—

A perfectly clear solution of bone marrow extract at pH 7.4 was divided into two portions. One portion was retained as control; the other, 5 cc., was placed inside a cellophane bag containing several glass beads. The bag was immersed in 5 cc. of saline at the same pH, and allowed to stand in a closed test tube at room temperature for 3 days, with agitation about once an hour during the day. The dialysate (that is, the solution outside the bag) was saved, and the bag was then subjected to further dialysis in a rocking dialyzer, against running buffered saline of the same pH. This was continued for 2 hours to remove any further dialyzable material. Previous tests had shown that with the rocking dialyzer used, half-saturated ammonium sulfate was rendered negative to Nessler's reagent in less than this period. 2 hours' dialysis against running saline, supervening on the previous 3 days, was deemed adequate to remove any dialyzable material from within the bag. The dialyzed solution as well as the undialyzed control was then diluted and injected into guinea pigs. The dialysate was injected undiluted.

In Table VII the results of this experiment are presented. It is apparent that after 3 days of simple dialysis no evidence of passage of the active agent through the membrane into the dialysate could be found. Furthermore, after additional dialysis against running saline, there was no diminution of activity of the dialyzed material compared with undialyzed controls. The conclusion may be drawn that the active agent is non-dialyzable.

Precipitation with Ammonium Sulfate.—Several experiments were carried out in which, to clear saline solutions of the active agent, calculated amounts of crystalline ammonium sulfate were added to produce varying degrees of saturation with this salt at room temperature. The precipitates were collected and redissolved, and after dialysis, precipitate and supernatant were separately injected into guinea pigs. At 0.3 saturation, no precipitate occurred. At 0.4 saturation, there was slight precipitate, which contained

a barely detectable trace of activity. The supernatant remained active. At 0.8 saturation, there was copious precipitate, which was active, while the supernatant fluid showed no residual activity. At 0.5 saturation the activity was quite equally divided between the supernatant and the precipitate. Table VIII is an example of one of these experiments, with half-saturated ammonium sulfate. Further work is required to determine more accurately the optimum range of percentage saturation with ammonium

TABLE VII

Effect of Dialysis on Active Agent

Inoculum	Dilution	Guinea pig No.	Results after 9 days	
			Clinical	Pathological
Dialyzed solution	1:4	1	+	++
		2	+	++
		3	±	++
	1:1	4	+++	+++
		5	++	+++
		6	+++	+++
Dialysate	1:1	7	0	0
		8	±	0
		9	0	0
Control solution, undialyzed	1:4	10	0	0
		11	+	++
		12	+	++
	1:1	13	++	+++
		14	++	+++
		15	+++	++

sulfate for the isolation of the active agent. The range lies roughly between 0.4 as the lower limit, and somewhat less than 0.8 as the upper.

Immune Reactions.—Although from the literature no unequivocal evidence of immunity to the active agent is forthcoming, a preliminary experiment gave suggestive results. 2 guinea pigs received 3 weekly subcutaneous injections of extract of proved potency and then were tested by intracerebral inoculation. The 2 animals showed typical symptoms, but survived. 2 controls died. This experiment was repeated 3 times, with results shown in Table IX. It can be seen that Experiment 2 is suggestive, for 4 animals which received the subcutaneous injections survived the intracerebral inoculation, while the controls died. However, this result was obtained on only one out of three attempts. As shown in the second

TABLE VIII

Effect of Half-Saturations with Ammonium Sulfate (and Dialysis) on Solution of Active Agent

Inoculum	Dilution	Guinea pig No.	Results after 8 days	
			Clinical	Pathological
Supernatant	1:4	1	0	+
		2	0	0
	1:1	3	++	+++
		4	++	+++
Sediment	1:4	5	0	0
		6	0	0
	1:1	7	+	++
		8	+	+++
Dialyzed control	1:4	9	+	+
		10	+	++
	1:1	11	+++	++++
		12	+++	++++

TABLE IX

Attempted Production of Immunity by Subcutaneous Inoculation of Agent

Experiment No.	No. of subcutaneous inoculations	Immunity test	
		Injected guinea pigs	Controls
1	3	7, 7, 8, 10	6, 7, 7, 9
2	4	S, S, S, S	6, 6, 7, 7
3	6	7, 8, 9, 10, 11	8, 8, 10, 10

7, 7, 8, 10 = guinea pigs died in 7, 7, 8, or 10 days, respectively.
S = survived. Killed 28 days after test inoculation.

Condition of Survivors of Experiment 2 (above), Examined 4 Weeks after Inoculation

Guinea pig No.	Symptoms	Pathology
1	±	+++
2	+	+++
3	++ to +++	+++
4	+ to ++	++++

part of Table IX, the survivors of Experiment 2 all showed marked brain damage, although their clinical signs had regressed. The subcutaneous in-

jections did not prevent the severe pathological damage, the essential feature of the disease, which results from intracerebral inoculation. Extensive experience with guinea pigs has shown that the degree of cellular damage is the criterion of the effect of the agent; death or survival are of secondary importance. Consequently these data are presented as negative results, in agreement with those of Edward (13), who worked with intravenously injected rabbits.

COMMENT

From the data presented above, it seems probable that the active agent is either a protein or intimately associated with a protein. There is no evidence, either in these experiments or in the literature, which is inconsistent with this viewpoint. It would obviously be desirable to attempt isolation and purification of the agent. For such a purpose more abundant material than is furnished by monkeys is essential. The spleen and lymph nodes of a single active case of Hodgkin's disease would be adequate for considerable progress. The data presented in this paper furnish indications of preliminary procedures that may advantageously be carried out for this purpose.

One of the chief significant features of the agent is its selective attack on nerve cells, namely, the Purkinje cells of the cerebellum. This action is similar to that produced by the virus of louping ill. It would be of great interest to discover the relation between a virus and a normal body protein, both producing an identical effect. The other factors of this relationship are discussed elsewhere (16).

SUMMARY

The nature of the encephalopathic agent present in monkey bone marrow has been investigated. It is readily soluble at a pH of 7.4, as well as at the low pH of 2.0 and 3.0. The point of minimal solubility is pH 4.2. Heat inactivation is produced by a temperature of 75°C., acting for 15 minutes. The agent is digested by pepsin. It exhibits a slight degree of sedimentation with the air-driven centrifuge. It is non-dialyzable. It is precipitated from solution (pH 7.4) by ammonium sulfate, over a range of 0.4 to 0.8 saturation. These findings all suggest that the agent is a protein. No immunological reactions could be elicited.

BIBLIOGRAPHY

1. Gordon, M. H., *Brit. Med. J.*, 1933, **1**, 641.
2. King, L. S., *J. Exp. Med.*, 1939, **70**, 303.
3. Gordon, M. H., *Brit. Med. J.*, 1934, **1**, 635.

4. Friedemann, U., and Elkeles, A., *Brit. Med. J.*, 1933, **2**, 1110.
5. Friedemann, U., *Brit. Med. J.*, 1934, **1**, 517.
6. Van Rooyen, C. E., *Edinburgh Med. J.*, 1937, **44**, 455.
7. Turner, J. C., Jackson, H., Jr., and Parker, F., Jr., *Am. J. Med. Sc.*, 1938, **195**, 27.
8. McNaught, J. B., *J. Am. Med. Assn.*, 1938, **111**, 1280.
9. Edward, D. G. ff., *Lancet*, 1938, **1**, 936.
10. MacKenzie, I., and Van Rooyen, C. E., *Brit. Med. J.*, 1935, **1**, 406.
11. Uhlenhuth, P., and Wurm, K., *Klin. Woch.*, 1936, **15**, 1025.
12. Van Rooyen, C. E., *Brit. Med. J.*, 1934, **1**, 519.
13. Edward, D. G. ff., *J. Path. and Bact.*, 1938, **47**, 481.
14. Kelser, R. A., and King, L. S., *Am. J. Path.*, 1936, **12**, 317.
15. Stanley, W. M., *Phytopathology*, 1934, **24**, 1055.
16. King, L. S., *J. Am. Med. Assn.*, 1939, **113**, 1940.

EXPLANATION OF PLATE 29

FIG. 1. A normal cerebellum, showing the even distribution of Purkinje cells. × 35.3.

FIG. 2. Damage to cerebellum from intracerebral injection of monkey bone marrow extract. The Purkinje cells have almost entirely disappeared and have been replaced by a vigorous glial reaction. In the areas indicated by arrows, a few Purkinje cells still persist intact. This is an example of a ++++ or maximal reaction. × 35.3.

1

2

Photographed by J A Carlile

(King Encephalopathic agent in primate bone marrow)

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PRIMARY ENCEPHALOMYELITIS IN GOATS ASSOCIATED WITH LISTERELLA INFECTION

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Bacterial infections of the nervous system generally take the form of a meningo-encephalitis or of focal metastatic encephalitis clearly secondary to infection elsewhere in the body. Primary encephalitis, from which a specific bacterium may almost constantly be recovered, raises new problems in neuropathology and warrants detailed consideration.

The bacterium in question, belonging to the genus *Listerella*, has a wide host range. It was first isolated in 1926 by Murray, Webb and Swann[1] from rabbits which were sick with mononucleosis. The name *B. monocytogenes* was applied. Independently Pirie[2, 3] in South Africa recovered a similar organism from a native rodent, the gerbille. Because the lesions were predominantly in the liver, he designated the bacterium as *Listerella hepatolytica*. The generic name *Listerella* has been finally adopted for this class of organisms.

That this new genus was of importance in human pathology soon became apparent. Burn,[4, 5, 6] and Schultz and his coworkers[7, 8] independently recovered unusual bacteria from human cases of meningitis. At first the identification was difficult, but finally conclusive evidence was brought forward to classify them with the *Listerella* group. Other papers[9, 10, 11] have since appeared in the literature reporting new cases of meningitis in which the same organism was found. It is probable that the cases reported by Atkinson[12] in 1917 were of the same type.

In addition, this bacterium has been recovered from human cases of infectious mononucleosis.[13, 14] Schmidt and Nyfeldt[15] have reported a series of 5 cases in which infectious mononucleosis and meningitis have coexisted. In 4, spinal fluid cultures revealed *Listerella*. In 1, there was also a positive blood culture.

As yet, in human nervous system involvement, only meningitis has been attributed to the *Listerella*. In lower animals, however, many cases of encephalitis have been observed from which the organism was recoverable in pure culture. Jones and Little[16] reported sporadic encephalitis in cows.

A comparable disease in sheep has been studied by Gill,[17, 18] by Jungherr,[19] and by Biester and Schwarte.[20] TenBroeck (unpublished) observed similar cases in sheep and goats. In addition, TenBroeck (also unpublished) recovered *Listerella* from chickens which showed no nervous system involvement, but instead myocardial necrosis.

Biological and immunological properties of *Listerella* have been studied by Seastone,[21] and by Webb and Barber,[22] and Barber,[23] as well as by other authors to whom reference has been made. These aspects of the problem will not be considered at the present time.

Material

The purpose of the present communication is to describe in detail the pathological findings in a series of 9 goats which were first studied by TenBroeck and Seastone in 1936. Although their observations have not been published, the pathological material has been placed at my disposal, as well as the clinical and bacteriological reports. The following data are drawn from their unpublished records.

These 9 animals all came from a single farm in New Jersey. They were taken ill during January, February and March, 1936, and brought to this laboratory for examination. During the same period several other goats of the same flock died with comparable symptoms, but were not autopsied or studied from the bacteriological standpoint.

The clinical course of the disease, lasting 2 or 3 days, showed nothing distinctive. The animals were obviously sick, with moderate fever, and reacted as in any severe infection. They would be down, unable to rise, but specific obvious neurological signs were rare. Specific motor weakness or spasticity was noted only infrequently.

Of the 9 animals studied, pure cultures of a Gram-positive organism were obtained from the brains of 6. In 1 other, the blood gave a positive culture although the brain did not. In 2 animals all cultures were negative. The organisms recovered were identified as belonging to the genus *Listerella*. Since the animals all came from a single flock and since the clinical course and the pathology were the same in every instance, there can be no reasonable doubt that the animals with negative cultures suffered from the same disease.

Paraffin embedded blocks of various portions of the nervous system, as well as of other organs, were obtained. For study hematoxylin-eosin and toluidine blue stains were made, and appropriate methods for demonstration of myelin, axis cylinders, reticulum and bacteria were employed. Because of the absence of unembedded tissue, fat stains could not be done.

Pathology

In the central neuraxis the lesions are restricted almost exclusively to the brain stem, especially the medulla and spinal cord. The disease process is much milder in the midbrain, while in the thalamus merely a cuffed blood vessel may occasionally be found. The basal olfactory regions may rarely show slight involvement. The neocortex and cerebellum are not affected.

The typical lesion consists of a small, compact, focal collection of cells which may be situated in white or gray matter. Examples in the spinal cord and medulla are seen under low power in Figures 1 and 2. These foci vary considerably in size. In serial sections cut at 15μ, single collections of cells may vary from 0.075 mm. to over 1 mm. in greatest dimension. Although the smaller foci are approximately spherical, those that extend over many sections are asymmetrical, the greatest dimension or length (usually running rostrocaudally) ranging up to ten times the cross section.

When examined under higher power the composition of these foci can be studied. Usually, though not always, there are a few polymorphonuclear leukocytes scattered sparsely among the other elements, but the majority of cells are always mononuclear. In favorable situations several different cell types may be clearly recognized. Numerous microglia may be identified by the abundant chromatin and irregular shape of their nuclei as well as by their typical branching cytoplasm, excellently brought out by toluidine blue. Even more numerous are the microglia-like cells, probably derived from the vascular adventitia. Typical oligodendroglia, with round compact nuclei having dense conspicuous chromatin and with scanty cytoplasm, are also clearly recognizable. Very prominent are larger, pale, oval or kidney shaped nuclei with scanty chromatin content. Such cells are provided with a moderate amount of compact cytoplasm, frequently round or oval, sometimes slightly angular, or with small, blunt, tuberous processes. These may be called epithelioid cells, for they are comparable to similar cells observed in well recognized granulomatous lesions of the brain. Astrocytes, easily recognizable in routinely stained sections of the nervous system, are not a component of these lesions.

In Figures 5 and 6 are seen examples of such circumscribed nodules under moderate enlargement. The different types of nuclei are apparent although the cytoplasmic details are not very clear in the reproduction. Also, in Figure 5 may be seen the syncytium formed by the cells and characteristic of many of these nodules. This syncytium is apparently similar to what Lillie[24] has described in the brain in Rocky Mountain spotted fever cases. These small foci or granulomas are usually in close associa-

tion with blood vessels although serial sections are necessary to establish the relationship. With such series it can be observed that the vessels may pass either directly through the mass of cells, or are in contact with it on one side. In the larger foci followed serially, more than one blood vessel can be seen in close apposition, and frequently the cell mass is larger in proximity to the vessels, smaller in the intervening regions. Sometimes, however, in spite of serial sections, a vascular relationship cannot be determined for every focus.

In many instances, especially where the cellular foci are less compact, a streaming of cells from the adventitia of blood vessels can be seen. Such *streaming is also observable in relation to pial surfaces of the nervous system. The cells involved are mononuclear and show a considerable range of morphology.* Some are similar to the so-called "active" and "transitional" microglia. Others have large vesicular nuclei and resemble the epithelioid cells.

The blood vessels show considerable alteration. Pronounced perivascular cuffing is readily observable in the low power illustrations, and may even occur in portions of the brain stem where focal nodules are absent. The coats of the blood vessels are profoundly disturbed. The endothelium may be swollen and proliferated. At other times it is degenerated and hyalinized. Small thrombi are not infrequent. The media may show marked disorganization and infiltration, with practically no structure remaining except infiltrating cells in an irregular hyaline matrix. Polymorphonuclear as well as mononuclear cells occur in the infiltrations. Figure 7 illustrates the disorganization of structure and infiltration of one vessel. It is noteworthy that perivascular hemorrhages have not been observed.

Where the disease process is advanced the parenchyma shows not only vascular and focal pathology, but also more diffuse and confluent changes. Rarely there may be small abscess formation, where dense masses of polymorphonuclear leukocytes, mingled with a few mononuclear cells, replace tissue that is practically destroyed. More typically, however, the separate foci become quite intense and the intervening tissue is more lightly infiltrated. Figure 3 shows under low power a Nissl stain of the medulla of 1 animal. Separate foci are large and there is moderately dense tissue infiltration that is diffuse. Such infiltration, however, is predominantly mononuclear and composed of the same types of cell that are found in the nodules. Figure 11 shows such a diffuse tissue infiltration under higher power. In such instances the parenchyma is not too severely damaged, incomparably less so than in the small abscesses. Figure 4 is a myelin stain of the section adjacent to that shown in Figure 3, and taken at the

same magnification. There are several, small focal areas of myelin destruction corresponding to the intense focal collections of cells. But the diffuse tissue infiltration (Fig. 11) is accompanied only by a mild lightening of the myelin, observable chiefly on the upper part of the figure.

Foci of demyelination show interesting features when examined under higher power. Figure 9 is taken from a myelin stained cross section of the spinal cord and shows a portion of the lateral column. In the center of the figure the clear space corresponds to a focal collection of cells such as is shown in Figure 5. Remnants of myelinated fibers persist, with here and there large ballooned sheaths. These ballooned fibers correspond in Figure 5 to the round spaces surrounded by cytoplasmic rims. The axis cylinders are present in these demyelinated areas to a slightly greater extent than the persisting myelin sheaths. In general the impression obtains that the fibers are pushed aside and choked out by the compact mass of cells, rather than undergoing any acute active or primary destruction.

The nerve cells in affected areas may or may not show changes. The striking feature is the paucity of morphological alterations that are visible in spite of the close proximity of large focal infiltration. But at times the nerve cells may be severely damaged or necrotic. In Figure 11, in the upper right corner, is one such injured neuron, with severe central loss of Nissl granules, but without swelling. Other cells, not figured here, may show more typical severe cell disease of Nissl. Increase of satellite glia cells is frequent, and numerous examples have been observed of necrotic nerve cells being actively phagocytosed by polymorphonuclear leukocytes. Pictures such as Figures 3 and 4 from a paper by Hurst,[25] taken from a case of virus encephalitis, could be accurately reproduced from the present goat material. Nuclear inclusions have not been observed.

In the majority of cases bacteria may be demonstrated somewhere in the diseased tissues, although by no means in every separate lesion. The morphological character of the bacteria as they appear in fixed tissue is adequately dealt with by previous workers and need not be repeated here. Figure 8 illustrates a marked degree of bacterial invasion. This particular animal was killed by chloroform and autopsied immediately, so that there was no opportunity for postmortem growth. In other lesions and in other animals fewer bacteria would be demonstrable in a field of comparable size. In a single oil immersion field of any given inflammatory or granulomatous lesion, bacteria, if present, would usually range up to a dozen in number, sometimes free in the tissue, usually phagocytosed by mononuclear cells. Bacteria were never demonstrable in the endothelium or walls of the blood vessels.

Meningeal involvement by the inflammatory process is common, but is generally slight in extent and secondary to parenchymal damage. Much less common are areas of meningitis where the underlying parenchyma is essentially free of lesions. Such an instance is seen in the lower part of Figure 10.

The disease process is not limited to the central nervous system or the leptomeninges. In one instance (Fig. 10) a well marked nodule was present in the dura over the spinal cord. Unfortunately, most of the sections do not include the pachymeninx, so that the frequency of this locus cannot be fairly estimated. That it may occur, however, is noteworthy.

In the peripheral nervous system lesions are seen occasionally in the cranial ganglia or nerves. Figure 12 illustratesa portion of the gasserian ganglion and associated fifth nerve fibers in one instance. Several other similar examples are available, although such peripheral location is by no means constant. In the illustration there is an intense infiltration of the body of the ganglion, chiefly by mononuclear cells. The infiltration is largely sharply focal and nodular, although the process is diffuse in some areas. The ganglion cells themselves show surprisingly little evidence of damage Most noteworthy, however, are the focal nodules in the nerve bundles. Under higher power these cell collections are indistinguishable from similar nodules in the cord and medulla, already described above.

Apart from the nervous system there is no constant pathology, although abnormalities are found. Nematodes in the lung are probably not related to the disease picture. Of greater relevance is the frequent involvement of the liver, either in the form of fatty infiltration or, less frequently, scattered focal necrosis. In 3 instances the kidneys show a definite glomerulitis. Cardiac abscesses were not noted.

DISCUSSION

The distribution of lesions is essentially restricted to the brain stem, especially the medulla and the spinal cord. The neocortex is entirely spared. Not only is there no injury to the parenchyma, but even the overlying meninges are not affected. So far as can be determined the cerebellum too is free of lesions, although blocks of this organ were not available in every instance. There are no data bearing on the cause of this predilection for the brain stem.

The primary lesion in the parenchyma is a circumscribed focal collection of mononuclear cells (with or without an admixture of a few neutrophilic leukocytes) in close relation to a blood vessel. The vessels show marked degenerative change, coupled with infiltration of the walls and adventitia. Diffuse cellular infiltration of nerve parenchyma and the

occasional formation of small abscesses are secondary as well as late phenomena.

The small granuloma-like lesions and the blood vessel changes suggest a similarity to the lesions seen in the brain in typhus,[26, 27] Rocky Mountain spotted fever,[24, 28, 29] and toxoplasmosis.[30, 31] This bacterial encephalitis is, however, much more fulminating and is accompanied by vastly more perivascular infiltration. But the focal granulomatous lesions are more similar to Rickettsial encephalitides than to virus infections of the nervous system.

Organisms of the genus *Listerella* have a very wide host range in nature and produce a variety of unrelated diseases. Thus, necrosis of liver and spleen, cardiac abscesses, meningitis, encephalitis and mononucleosis occur in different hosts, while the causative organism in each case is very closely related. Experimentally all these separate conditions can be reproduced, with the exception of encephalitis.

In reference to encephalitis, the causal rôle of the organisms is still somewhat obscure. Although the specific bacterium may be recovered from the brain in pure culture, Koch's postulates cannot be fulfilled, since the disease is not accurately reproducible by inoculation. In the cases reported here, TenBroeck (unpublished) injected pure cultures of *Listerella* intracerebrally into sheep and goats. The inoculated animals died, but the disease process was a meningitis and meningo-encephalitis, not a true encephalitis as has been described above.

In the literature similar difficulties are reported. Gill,[18] who isolated bacteria from the brains of sheep, on re-inoculation intracerebrally into normal sheep could produce only meningo-encephalitis. Inoculation into sheep by intracarotid injection or intranasal instillation similarly produced meningitis. Whatever brain involvement was present was not comparable to the naturally occurring disease, although suggestive evidence was obtained after intracarotid injection.

Jungherr,[19] attempting the experimental induction of the natural disease in sheep, was also unsuccessful. In mice, in a few instances, after nasal instillation of the bacteria, he was able to find typical nodules in the medulla, but he concludes that the pathogenesis of the natural disease is not understood, although *Listerella* must play an important rôle as an etiological agent. On the other hand, Biester and Schwarte[20] believe that they reproduced the natural disease by inoculation, but they present no evidence on the subject.

Burn,[5] with organisms isolated from human cases of meningitis, produced purulent meningitis in rabbits after intravenous inoculation. Of 4 monkeys injected intravenously, 2 showed meningitis alone, a 3rd animal exhibited

marked infiltration of the parenchyma of the cortex with polymorphonuclear leukocytes, while the 4th animal was not affected. This single instance of parenchymal involvement in a monkey is most unusual, for it resembles neither the human disease nor the encephalitis in lower animals.

Factors of host peculiarity are excellently brought out by Burn. Intravenous inoculation of *Listerella* into rabbits results in meningitis. Similar inoculation into guinea pigs causes myocardial abscesses without affection of the meninges. But intraperitoneal injection into guinea pigs results in meningitis without myocardial abscesses.

Gibson[9] inoculated rabbits and guinea pigs with *Listerella* isolated from human meningitis but was not able to reproduce either encephalitis or meningitis. Similarly Seastone,[21] working with organisms recovered from chickens, could not induce primary meningitis or encephalitis in rabbits or guinea pigs by intravenous injections. However, after profound systemic infection the nervous system showed a slight reaction.

It is clear from the literature that experimental meningitis can be produced quite regularly by subdural injections of *Listerella,* as well as less constantly by other routes of inoculation. Encephalitis, however, has not been satisfactorily demonstrated by experimental methods in spite of occasional suggestive instances. Obviously there is some additional factor in the naturally occurring cases of encephalitis which eludes experimental analysis. The suggestion has been made that in addition to the bacteria a virus is involved in the pathogenesis, so that the natural encephalitis is a mixed infection. However, attempts to demonstrate a separate virus have not been successful. The problem for the present remains unsolved. In the natural disease, in goats as well as in sheep and cows, the invasion of the brain parenchyma by bacteria appears to be a primary process, independent of any discoverable focus of infection elsewhere in the body.

SUMMARY

In a series of 9 goats spontaneous encephalitis associated with *Listerella* infection was observed.

Pathologically the lesions were essentially restricted to the brain stem, especially the medulla and spinal cord. Peripheral nerves and ganglia were inconstantly affected. There was an associated meningitis.

The primary parenchymal lesion is a circumscribed focal collection of mononuclear cells (with or without an admixture of a few neutrophilic leukocytes) in close relation to a blood vessel. The infection is very fulminating. Diffuse tissue infiltration may supervene, sometimes with the formation of small abscesses, but these are secondary and late phenomena. The blood vessels show marked degenerative changes, coupled with cellular infiltration of the walls and adventitia. Bacteria can frequently be demon-

strated histologically in the parenchymal lesions. Nerve cells may be destroyed, but there is relatively little tissue necrosis.

Data on experimental transmission and pathogenesis are briefly discussed. The similarity to Rickettsial encephalitides and cerebral toxoplasmosis is pointed out.

REFERENCES

1. Murray, E. G. D., Webb, R. A., and Swann, M. B. R. A disease of rabbits characterised by a large mononuclear leucocytosis caused by a hitherto undescribed bacillus *Bacterium monocytogenes* (n. sp.). *J. Path. & Bact.*, 1926, **29**, 407–439.
2. Pirie, J. H. H. Bacterial encephalitis from the genus *Listerella*: a new disease of man and animals. *South African M. J.*, 1938, **12**, 51.
3. Pirie, J. H. Harvey. A new disease of veld rodents, "Tiger River disease." *Publ. South African Inst. Med. Research*, 1927, **3**, 163–186.
4. Burn, Caspar G. Unidentified Gram positive bacillus associated with meningo-encephalitis. *Proc. Soc. Exper. Biol. & Med.*, 1934, **31**, 1095–1097.
5. Burn, C. G. Characteristics of a new species of the genus *Listerella* obtained from human sources. *J. Bact.*, 1935, **30**, 573–591.
6. Burn, Caspar G. Clinical and pathological features of an infection caused by a new pathogen of the genus *Listerella*. *Am. J. Path.*, 1936, **12**, 341–348.
7. Schultz, E. W., Terry, M. C., Brice, A. T., Jr., and Gebhardt, L. P. Bacteriological observations on a case of meningo-encephalitis. *Proc. Soc. Exper. Biol. & Med.*, 1933–34, **31**, 1021–1023.
8. Schultz, E. W., Terry, M. C., Brice, A. T., Jr., and Gebhardt, L. P. *Listerella monocytogenes*: a cause of meningo-encephalitis in man. *Proc. Soc. Exper. Biol. & Med.*, 1938, **38**, 605–608.
9. Gibson, H. J. A pathogenic diphtheroid bacillus from a fatal case of meningitis. *J. Path. & Bact.*, 1935, **41**, 239–252.
10. Carey, Benjamin W., Jr. Infection with an organism of the genus *Listerella*; report of a case of acute cerebrospinal meningitis with recovery. *J. Pediat.*, 1936, **8**, 626–629.
11. Wright, Helen A., and Macgregor, Agnes R. A case of meningitis due to *Bacterium monocytogenes*. *J. Path. & Bact.*, 1939, **48**, 470–472.
12. Atkinson, Everitt. Meningitis associated with Gram-positive bacilli of diphtheroid type. *M. J. Australia*, 1917, **4**, 115–118.
13. Nyfeldt, Aage. Etiologie de la mononucléose infectieuse. *Compt. rend. Soc. de biol.*, 1929, **101**, 590–592.
14. Pons, C. A., and Julianelle, L. A. Isolation of *Listerella monocytogenes* from infectious mononucleosis. *Proc. Soc. Exper. Biol. & Med.*, 1939, **40**, 360–361.
15. Schmidt, Viggo, und Nyfeldt, Aage. Ueber Mononucleosis infectiosa und Meningo-encephalitis. *Acta oto-laryng.*, 1938, **26**, 680–688.
16. Jones, F. S., and Little, Ralph B. Sporadic encephalitis in cows. *Arch. Path.*, 1934, **18**, 580 (abstract).
17. Gill, D. A. "Circling" disease of sheep in New Zealand. *Vet. J.*, 1931, **87**, 60–74.
18. Gill, D. A. Circling disease: a meningo-encephalitis of sheep in New Zealand. *Vet. J.*, 1933, **89**, 258–270.
19. Jungherr, Erwin. Ovine encephalomyelitis associated with *Listerella* infection. *J. A. V. M. A.*, 1937, N.S. **44**, 73–87.

20. Biester, H. E., and Schwarte, L. H. Studies on *Listerella* infection in sheep. *J. Infect. Dis.*, 1939, **64**, 135–144.
21. Seastone, C. V. Pathogenic organisms of the genus *Listerella*. *J. Exper. Med.* 1935, **62**, 203–212.
22. Webb, Robert A., and Barber, Mary. *Listerella* in human meningitis. *J. Path. & Bact.*, 1937, **45**, 523–539.
23. Barber, Mary. A comparative study of *Listerella* and *erysipelothrix*. *J. Path. & Bact.*, 1939, **48**, 11–23.
24. Lillie, R. D. Pathology of the eastern type of Rocky Mountain spotted fever. *Pub. Health Rep.*, 1931, **46**, 2840–2859.
25. Hurst, E. Weston. The histology of equine encephalomyelitis. *J. Exper. Med.*, 1934, **59**, 529–542.
26. Wolbach, S. Burt, Todd, John L., and Palfrey, Francis W. The Etiology and Pathology of Typhus. Being the Main Report of the Typhus Research Commission of the League of Red Cross Societies to Poland. League of Red Cross Societies, Harvard University Press, Cambridge, Mass., 1922.
27. Spielmeyer, W. Die zentralen Veränderungen beim Fleckfieber und ihre Bedeutung für die Histopathologie der Hirnrinde. *Ztschr. f. d. ges. Neurol. u. Psychiat.*, 1919, **47**, 1–54.
28. Lillie, R. D., Dyer, R. E., and Topping, N. H. Cerebral pathology in rodents in endemic typhus and Rocky Mountain spotted fevers. *Pub. Health Rep.*, 1939, **54**, 2137–2148.
29. Pinkerton, Henry, and Maxcy, Kenneth F. Pathological study of a case of endemic typhus in Virginia with demonstration of Rickettsia. *Am. J. Path.*, 1931, **7**, 95–104.
30. Wolf, Abner, and Cowen, David. Granulomatous encephalomyelitis due to an encephalitozoon (encephalitozoic encephalomyelitis). *Bull. Neurol. Inst. New York*, 1937, **6**, 306–371.
31. Wolf, Abner, Cowen, David, and Paige, Beryl H. Toxoplasmic encephalomyelitis. III. A new case of granulomatous encephalomyelitis due to a protozoon. *Am. J. Path.*, 1939, **15**, 657–694.

DESCRIPTION OF PLATES

Plate 99

Fig. 1. Spinal cord in the early stage of the disease process with numerous focal collections of cells in both white and gray matter. The blood vessels are quite heavily infiltrated. Toluidine blue stain. × 17.5.

Fig. 2. Medulla oblongata in the early stage of the disease process, with discrete inflammatory nodules, many of which are in obvious relation to blood vessels. Toluidine blue stain. × 32.

Fig. 3. Cross section of the medulla in the late stage of the disease. The focal cell collections are intense and large, and there is considerable diffuse parenchymal infiltration. The blood vessels are very heavily cuffed. There are a few necrotic areas. Meningitis is insignificant. Toluidine blue stain. × 8.

Fig. 4. Section adjacent to that in Figure 3, but stained for myelin. The heavily cuffed blood vessels and the focal areas of necrosis stand out as clear spaces. The severe diffuse infiltration of tissue seen in Figure 3 is reflected only by a mild lightening in the myelin stain. × 8.

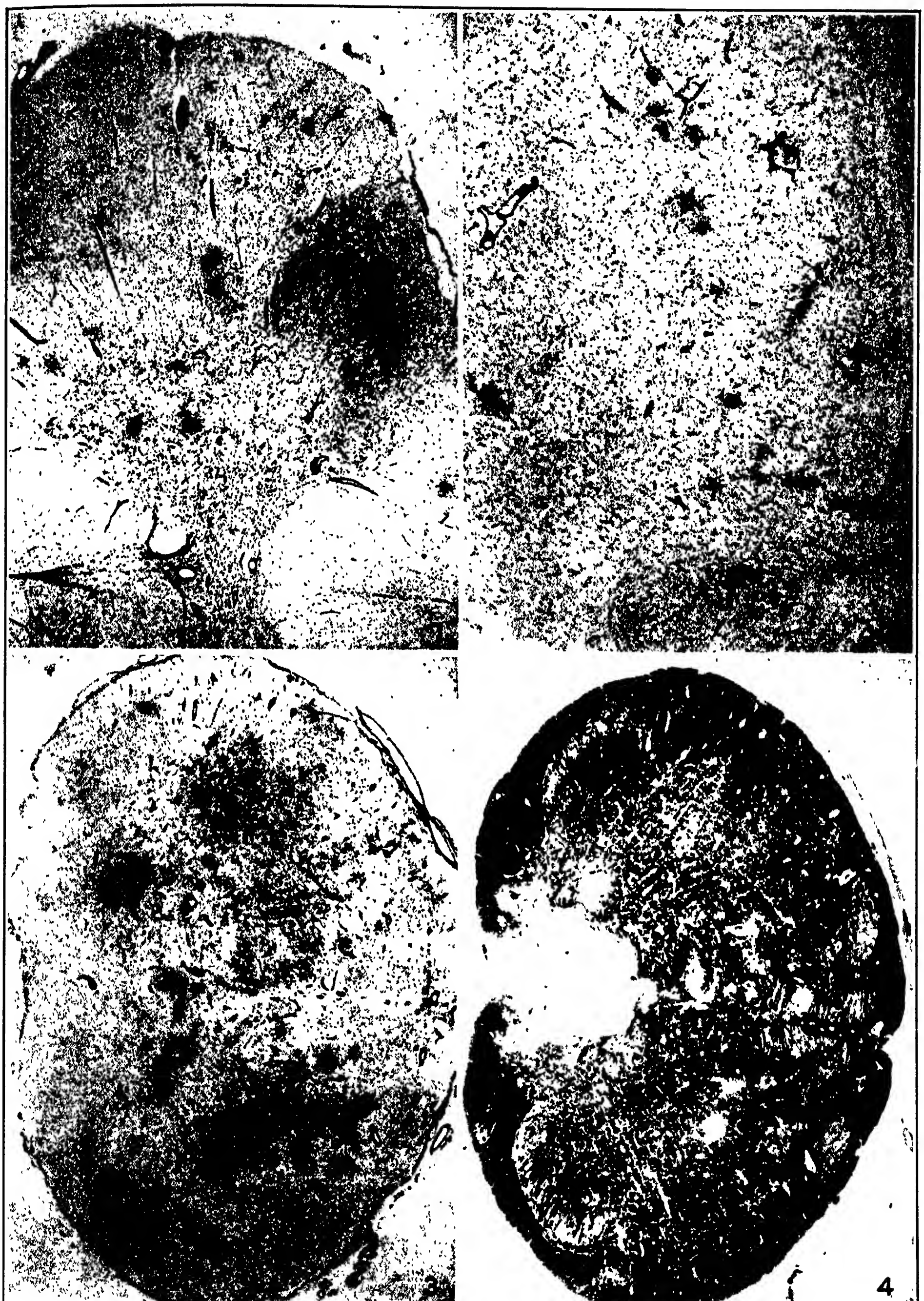
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Plate 100

Fig. 5. A focal collection of cells seen under higher power. There are a few polymorphonuclear leukocytes, but the majority are mononuclear, some glial, some derived from the blood stream or the vascular adventitia. A cytoplasmic syncytium can be seen. The large round spaces with cytoplasmic rims correspond to ballooned myelin sheaths. Toluidine blue stain. × 455.

Fig. 6. Another focal nodule, showing nuclei of epithelioid cells in addition to other elements. Toluidine blue stain. × 574.

Fig. 7. A blood vessel showing severe disorganization, necrosis and hyalinization, and marked infiltration with mononuclear and polymorphonuclear cells. Hematoxylin-eosin stain. × 550.

Fig. 8. Focus of Gram-positive bacteria in the parenchyma of the medulla. Gram-Weigert stain. × 1157.


5
6
7
8

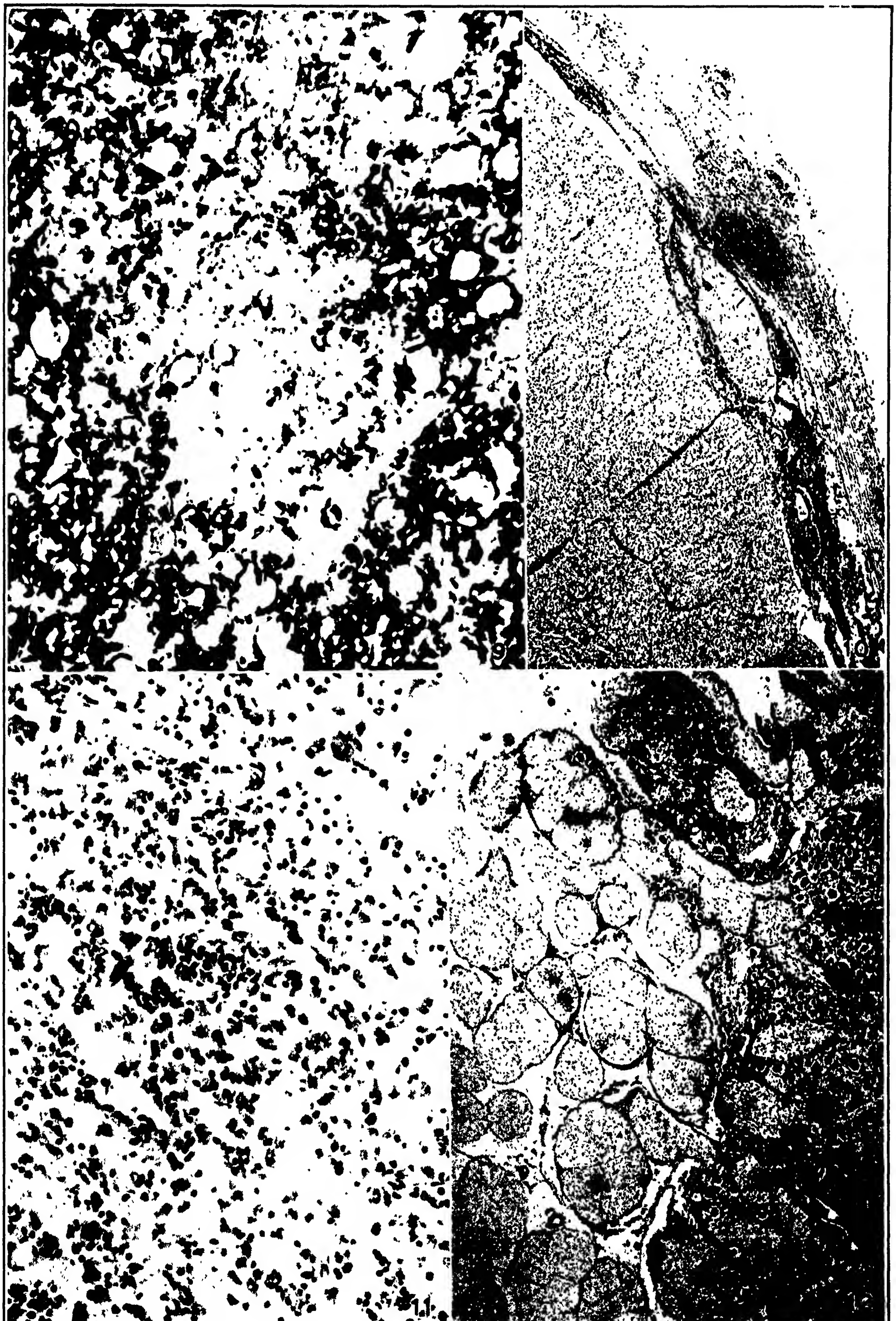
Plate 101

Fig. 9. A lesion in the white matter of the spinal cord, stained for myelin. The pale area, corresponding to the focal collection of cells, contains many fragmented myelin sheaths and myelin droplets. × 292.

Fig. 10. Intense cellular focus in the dura overlying the spinal cord. At the lower right there is intense meningitis, but no focal myelitis in the subjacent parenchyma. Hematoxylin-eosin stain. × 33.

Fig. 11. Diffuse tissue infiltration, predominantly mononuclear, in the medulla. There is a severely injured neurone in the upper right corner. Toluidine blue stain. × 240.

Fig. 12. Inflammation of the gasserian ganglion and fifth nerve fibers. The focal collections of cells in the nerve bundles on the left are entirely similar to those seen in Figures 5 and 6. Toluidine blue stain. × 33.



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THE BEHAVIOR OF POX VIRUSES IN THE RESPIRATORY TRACT

III. THE SURVIVAL OF VARIOLA AND VACCINIA VIRUSES IN THE LUNGS OF MICE PREVIOUSLY INFECTED WITH VARIOLA

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PLATE 30

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The comparative behavior of the single strains of vaccinia and variola virus studied by us in the mouse[1, 2] following nasal instillation was quite dissimilar. Vaccinia virus produced a systemic disease with well marked symptoms and a moderately high mortality, whereas variola virus was largely innocuous. The latter was regularly recoverable, however, from the lung through the 5th day and sometimes through the 7th day by transfer to embryonated eggs, and during its brief residence commonly gave rise to definite though rather inconspicuous pathological changes. The experiments here reported were carried out to determine whether variola virus elicited any protective response during its short period of survival in the lung.

Mice from a colony maintained at The Rockefeller Institute at Princeton and known to be conspicuously free of respiratory tract infections were employed in all of the following tests. The strain of variola virus was one isolated by us in 1938 and maintained thereafter by successive transfers in embryonated eggs.[2] In culturing the virus 10 day hen's eggs were inoculated by the Burnet method and incubated at 37°C. for 72 hours. The chorioallantoic membrane was removed aseptically, finely ground, and diluted with sufficient saline to make approximately a 10 per cent suspension. Membranes from a number of different transfers, the 97th through the 133rd, were used at one time or another in carrying out the protection tests. They were bacteriologically sterile and when removed on the 3rd day showed a typical reaction. Young mice weighing 15 to 20 gm. were etherized and infected by holding the nose for several seconds in the membrane suspension. On recovery from the anesthesia they were divided into groups of 15 and maintained under strict quarantine in an isolation unit. A few non-specific deaths occurred during the first week, but most of the mice were unaffected by the procedure and gained weight normally.

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[1] Nelson, J. B., *J. Exp. Med.*, 1938, **68,** 401.

[2] Nelson, J. B., *J. Exp. Med.*, 1939, **70,** 107.

One mouse from each group was killed on the 5th day following nasal instillation of the virus and autopsied. The lung was removed aseptically and examined under a low power microscope (6×). It was finely ground with a small amount of saline in a glass tissue grinder and sufficient diluent added to make roughly a 10 per cent suspension. Two 10 day embryonated eggs were inoculated with small, unmeasured amounts of each suspension and examined 3 days later. At autopsy the lungs of these mice showed a characteristic reaction and contained sufficient virus to produce lesions on the chorio-allantois of the inoculated eggs. Mice after they were given a second nasal instillation of variola virus were examined in the same way. A small piece of lung was removed for histological examination from some of the animals.

A few mice originally infected with variola were subsequently tested with vaccinia virus. The strain employed came originally from the New York City Board of Health and had been maintained for several years in an egg membrane suspension. All of these mice were infected intranasally with the third egg transfer of this strain. The same general technique was followed in carrying out the tests.

The Survival of Variola Virus in the Lungs of Mice Previously Infected with the Homologous Virus

Mice which had previously received a single nasal instillation of variola virus were reinfected by the same route after intervals of 7 to 27 weeks and killed 1 day, 3 days, and 5 days later. Normal mice of approximately the same age were included in each test as a control on the activity of the particular virus suspension employed. The results of these experiments are summarized in Tables I to IV.

Three of the 5 mice killed on the 1st day after reinfection showed no macroscopic pulmonary reaction, while the lungs of 2 contained small translucent areas visible only by low magnification. Variola virus was demonstrable in each of the 5 lung suspensions on inoculation in embryonated eggs. Judged by the number of foci in the chorioallantois the virus titer of only one suspension was significantly low. The mice in this experiment were reinfected 20 weeks after the primary nasal instillation.

Two mice of normal susceptibility were also killed 24 hours after they had been infected with variola. The lungs of both animals contained the specific virus, but only one showed a single small area of reaction.

Each of the 5 mice killed on the 3rd day after reinfection showed pulmonary changes, which varied from patchy discrete areas of abnormal opacity to involvement of one-third or more of several lobes. These mice were reinfected approximately 20 weeks after the initial introduction of virus. The pulmonary reaction was comparable to that of 2 normal mice injected with the same virus and killed 3 days later. Virus was present in the lungs of the 5 reinfected mice but in comparison with the mice of normal susceptibility and with similar ones previously killed on the 3rd day its titer was somewhat reduced. There was a marked reduction, however, in only one lung, the suspension of which produced 5 and 9 foci, respectively, in the chorioallantoic membranes of 2 embryonated eggs. Discrete foci varying from 25 to 100 were present in the egg membranes inoculated with the other 4 lung suspensions. The chorioallantoic foci produced by the lung suspensions from the normal mice were either too numerous to count or were semiconfluent.

Twenty-five mice were killed on the 5th day after reinfection with variola virus, the

interval between injections varying from 7 to 28 weeks. All of the animals in this group showed a moderate to a pronounced pulmonary reaction which was not significantly different macroscopically from that at 3 days. The lung suspensions from 21 of these mice were free of virus, no foci being produced on the chorioallantois of inoculated eggs. A second transfer was made from several of the membranes, with negative results. Three of the lung suspensions contained specific virus but in significantly low concentration, the inoculated egg membranes showing 3, 5, and 12 discrete foci, respectively. These 3 lung suspensions were from mice inoculated 21 to 27 weeks after the primary nasal instillation of virus.

TABLE I

The Examination of Recovered and Normally Susceptible Mice One Day after Nasal Instillation of Variola Virus

Type of mouse	Mouse No.	Interval between injections	Pulmonary reaction	Chorioallantoic reaction
		wks.		
Recovered	1	20	—*	Semiconfluent
	2	20	—	"
	3	21	+	50 foci
	4	21	—	3 "
	5	21	—	25 "
Normally susceptible	6		—	Semiconfluent
	7		±	Innumerable foci

* The symbols used are merely indicative of the degree of involvement.

TABLE II

The Examination of Recovered and Normally Susceptible Mice 3 Days after Nasal Instillation of Variola Virus

Type of mouse	Mouse No.	Interval between injections	Pulmonary reaction	Chorioallantoic reaction
		wks.		
Recovered	1	21	++	9 foci
	2	21	+++	25 "
	3	21	++	25 "
	4	21	++	50 "
	5	21	++	100 "
Normally susceptible	6		++	Semiconfluent
	7		+++	Innumerable foci

The sample of variola virus recovered on the 5th day from the lung of one of the reinfected mice (No. 21 in Table III) was carried through 3 subsequent egg transfers at intervals of 3 days. The chorioallantois from the original transfer contained 3 discrete foci, whereas the membranes from the later passages all showed the extensive confluent

thickening characteristic of a high concentration of the specific virus. There was no involvement of the embryo in any of the inoculated eggs. If the virus had undergone any modification as a result of its 5 day residence in the lung of a recovered mouse it was not demonstrable by the egg technique.

Lung sections were examined from 10 of the mice killed on the 5th day after reinfection. The only observed difference in the histopathology of these lungs and those

TABLE III

The Examination of Recovered Mice 5 Days after Nasal Instillation of Variola Virus

Mouse No.	Interval between injections	Pulmonary reaction	Chorioallantoic reaction
	wks.		
1 to 6	7–15	++ or +++	No foci
7 to 11	20	++ or +++	" "
12 to 20	21	++ or +++	" "
21	21	+++	3 "
22	21	+++	No "
23	28	+++	5 "
24	28	+++	No "
25	28	+++	12 "

TABLE IV

The Examination of Normally Susceptible Mice 5 Days after Nasal Instillation of Variola Virus

Mouse No.	Pulmonary reaction	Chorioallantoic reaction
1	+++	50 foci
2	+++	25 "
3	+++	125 "
4	+++	50 "
5	+++	25 "
6	–	20 "
7	++	Innumerable foci
8	+++	" "
9	+++	" "
10	+++	50 "

from mice which had not been previously infected was a quantitative one, the cellular reaction appearing to be more extensive. All of the sections from the reinfected mice showed wide cellular cuffs, composed chiefly of lymphocytes and mononuclear cells together with an occasional leucocyte, around blood vessels or in association with bronchioles. An increase in the number of mononuclear cells lying free in the alveolar spaces was also apparent. A characteristic picture of this cellular infiltration is shown in Fig. 5.

Ten normal mice injected intranasally with the same egg membrane passage of variola virus were also killed on the 5th day. Nine of these mice showed a pulmonary

reaction indistinguishable macroscopically from that of the reinfected animals. The lung of one mouse was normal. All of the lung suspensions contained virus, the titer varying considerably from mouse to mouse. The chorioallantoic foci in eggs inoculated from 3 of the suspensions were too numerous to count. Seven of the membranes contained well spaced discrete foci which varied in number from 20 to 125.

The appearance of the chorioallantoic membrane of eggs inoculated with lung suspensions from recovered and normal mice killed on the 5th day after nasal instillation of variola virus is shown in Figs. 1 and 2.

The Survival of Vaccinia Virus in the Respiratory Tract of Mice Previously Infected with Variola Virus

Six mice which had previously received a single nasal instillation of variola virus were infected intranasally 24 weeks later with vaccinia virus. After an incubation period of 3 days all of the mice showed symptoms of the systemic disease produced in mice by vaccinia on introduction into the upper respiratory tract. Unless disturbed, the mice huddled together at one end of the cage. There was a progressive loss of weight accompanied by weakness and an unkempt appearance. On the 5th day 3 of the mice began to chatter intermittently. One mouse was killed and autopsied at this time. The nasal passages were normal and elementary bodies were not demonstrable by the silver impregnation method of Morosow. The lung, however, was involved, 3 lobes showing an extensive reaction and containing virus. The concentration of virus was low, as only 10 discrete and widely separated foci were produced on the chorioallantois of an inoculated fertile egg. The 5 remaining mice were kept under observation until the 8th day when they were killed and autopsied. At this time they were all fairly normal in appearance and had begun to regain weight. There was a moderate macroscopic reaction in each of the 5 lungs but only one contained virus. The virus titer of this one suspension was very low, producing only 3 foci on egg inoculation.

Six normal mice of approximately the same age were infected intranasally at the same time with the same suspension of vaccinia virus. Judged by the behavior of these mice up to the 5th day, their response was similar to that of the mice previously infected with variola virus. One mouse was sacrificed at this time. At autopsy there was a definite rhinitis with demonstrable elementary bodies and a moderate reaction in the lung which contained a high concentration of virus, sufficient to produce a wide oval area of necrosis in the chorioallantois of an inoculated egg and to kill the embryo. The condition of the 5 remaining mice became progressively worse and was terminated by death on the 6th day. Egg inoculations were not made from the lungs of these mice but all of them showed an extensive involvement.

Five additional mice infected with vaccinia virus 20 weeks after the initial nasal instillation of variola virus were killed on the 5th day and examined. Characteristic symptoms were apparent beginning on the 3rd day. At autopsy the nasal passages were normal and elementary bodies were not demonstrable. All of the mice showed a typical involvement of the lung from which the specific virus was recovered by the inoculation of embryonated eggs. The virus titer of the lung suspensions was low. Three of the suspensions produced discrete foci which numbered 3, 50, and 100, respectively. Two of them produced isolated groups of coalesced foci. The embryos of these eggs were all normal and active on the 3rd day.

Five normal mice, the survivors of a group of 10 infected with the same egg transfer of vaccinia virus but a different suspension from that employed in the above experiment, were also killed on the 5th day. At autopsy these mice showed a specific rhinitis and a characteristic pulmonary involvement. The reaction in embryonated eggs inoculated with the lung suspensions indicated a high titer of virus. All of the suspensions produced a wide confluent area of hyperplasia and necrosis in the chorioallantois and 4 of them resulted in death of the embryo on the 3rd day.

Vaccinia virus reisolated from the lung of a mouse originally infected with variola and later with vaccinia was carried through two additional transfers in embryonated eggs. The initial inoculation, made directly from the lung, had no effect on the embryo and produced only 3 small discrete foci in the chorioallantoic membrane. With the two subsequent transfers the embryos were killed on the 3rd day and large oval areas of reaction were produced in the membrane. With respect to its behavior in fertile eggs there was no indication that the virus was affected by its 5 day residence in the lung of a mouse recovered from a previous infection with variola.

A total of 36 normal mice were infected with vaccinia by the nasal route as controls for the protection tests. Twenty-four of these mice died on the 3rd to the 8th day following nasal instillation of the virus. The mortality rate of 66 per cent is in close agreement with that reported in our earlier experiments on vaccinial infection following nasal inhalation, namely 72 per cent.[1]

The reaction produced in the chorioallantois of eggs by vaccinia virus present in lungs removed on the 5th day from normally susceptible mice and from mice which had recovered from a previous infection with variola is shown in Figs. 3 and 4.

DISCUSSION

The preceding observations indicate that the survival of variola virus in the lung of the mouse is influenced by recovery from a previous infection. In normally susceptible mice the virus is usually demonstrable in the lung through the 5th day and occasionally through the 7th day. In recovered mice there was no significant change in the amount of virus recovered from the lung 24 hours after nasal instillation, but by the 3rd day the titer was noticeably reduced and on the 5th day the lung was usually virus-free.

In mice of normal susceptibility variola virus produces a transient pulmonary reaction which is characterized chiefly by an accumulation of lymphocytes and mononuclear cells. It is presumably a defense mechanism in part, directed against removal or limitation of the virus. In reinfected mice the macroscopic pulmonary changes were similar through the 5th day, but microscopically a somewhat more extensive cellular reaction was noted. In these mice the earlier sterilization of the lung in respect to the specific virus may be brought about by the combined action of the mobilized phagocytic cells and some protective by-product of the initial infection.

The activity of vaccinia virus in the respiratory tract of the mouse is

also influenced by a previous infection with variola. The nasal instillation of vaccinia virus in normally susceptible mice is followed by a local multiplication leading to a systemic disease which may terminate fatally. The lungs of mice which live through the 5th day contain a high titer of the specific virus. In mice which had recovered from an earlier infection with variola the usual symptoms of the vaccinial reaction were present but they did not progress and by the 5th day the titer of virus in the lung was markedly reduced. Actual sterilization as in the case of variola was not effected. This incomplete action against vaccinia is in agreement with earlier observations in the monkey which indicated that variola virus produced only partial protection against vaccinia.[2, 3]

Variola virus being inactive in the mouse outside the lung, there was no indication from the experimental observations whether the defense mechanism operative against it in previously infected animals was purely local or the local response of a general reaction. Following nasal instillation in normally susceptible animals, vaccinia virus is also active in or on the mucous membrane of the upper air passages. Since the mice infected with vaccinia virus after recovery from variola showed no evidence of a specific rhinitis, it may be presumed that the defense reaction is generalized and attributable in part to a circulating antibody.

SUMMARY

Recovery from the transient pulmonary reaction which accompanies the nasal instillation of variola virus in mice was followed by a measurable protection against the homologous virus and also against vaccinia.

Variola virus which regularly survived in the lung of normally susceptible mice through the 5th day was noticeably reduced in titer on the 3rd day in the lung of recovered animals, and usually eliminated by the 5th day.

Vaccinia virus produced a less severe systemic reaction in recovered mice and its titer in the lung was significantly reduced on the 5th day.

The residence of both viruses in the lung of recovered mice was attended by pathological changes, visible macroscopically.

[3] Gordon, M. G., *Great Britain Med. Research Council, Special Rep. Series, No. 98*, 1925.

EXPLANATION OF PLATE 30

The unenlarged chorioallantois of embryonated eggs inoculated with the following lung suspensions from mice killed on the 5th day.

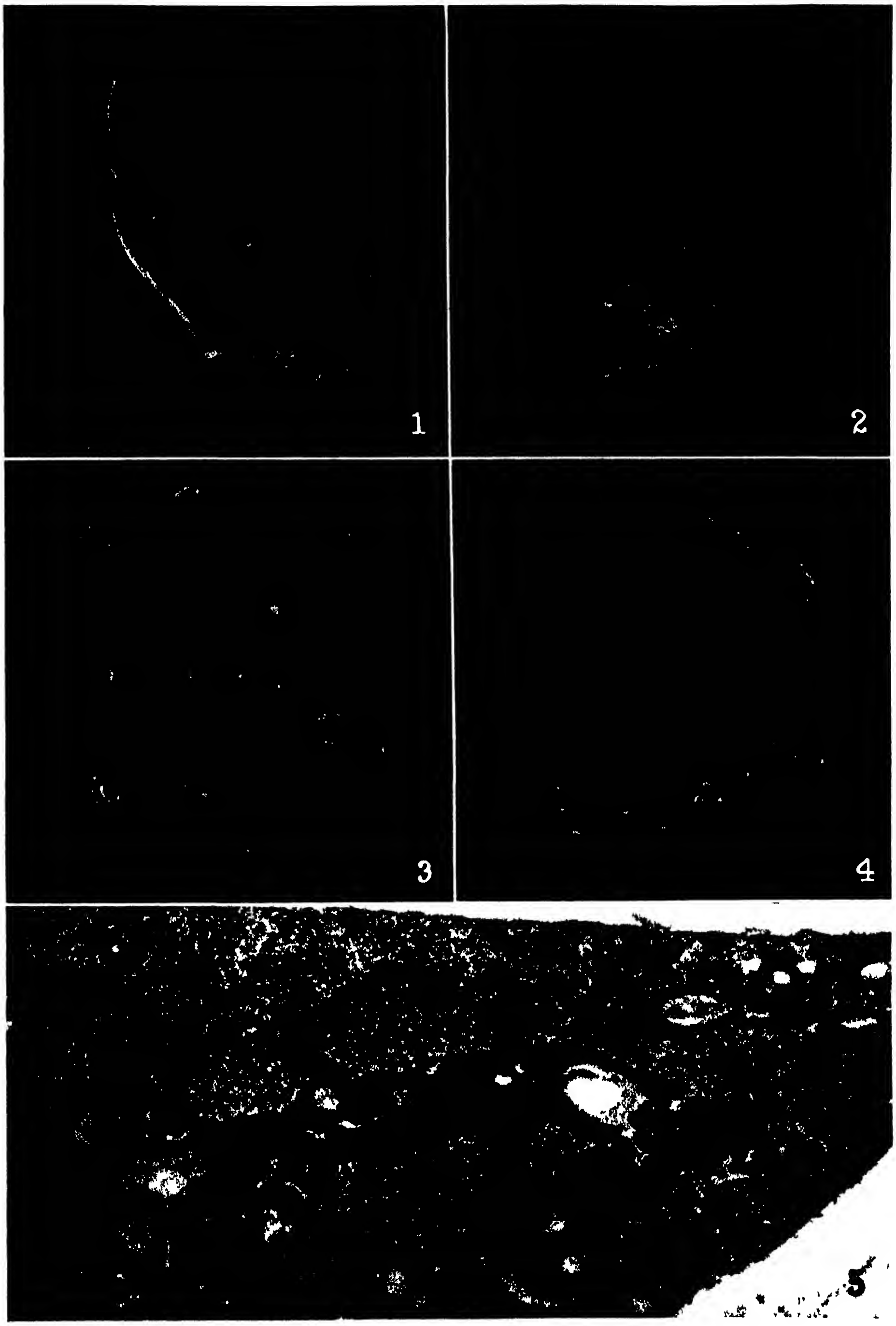
FIG. 1. Recovered mouse reinfected with variola virus, the membrane showing 3 discrete foci.

FIG. 2. Normally susceptible mouse infected with variola virus.

FIG. 3. Recovered mouse infected with vaccinia virus.

FIG. 4. Normally susceptible mouse infected with vaccinia virus, the membrane showing a wide area of confluent foci.

FIG. 5. Cellular infiltration in alveoli and around vessels in lung of a recovered mouse reinfected with variola and killed on the 5th day. Phloxin-methylene blue. × 46.



Photographed by J A. Carlile

(Nelson Pox viruses in respiratory tract III)

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THE CHEMICAL NATURE OF GROWTH FACTORS REQUIRED BY MOSQUITO LARVAE

II. Pantothenic Acid and Vitamin B_6

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When the larvae of the yellow fever mosquito, *Aedes aegypti*, are grown under sterile conditions (1), they may be shown to require accessory growth factors which they normally obtain from living microorganisms. Two of these factors are thiamin and riboflavin (2). If the larvae are supplied with protein, thiamin, and other dietary constituents in the form of a suitable amount of heat-killed yeast, they do not grow unless the yeast is further supplemented with adequate amounts of both riboflavin and a partially purified fraction of liver extract designated as the calcium filtrate (2). The experiments to be reported in this paper have been concerned with attempts to discover the nature of the essential substances present n the calcium filtrate fraction.

Methods

The larval growth tests were conducted in the manner previously described (2). All tubes received 0.3 ml. of a washed, killed yeast suspension in distilled water and either riboflavin or the flavin-purine complex (2). As formerly, all operations were carried out under strictly sterile conditions and appropriate tests for sterility were made. The growth index $N \times \frac{1}{T}$ (N = percentage of larvae reaching the 4th or last larval instar within 10 days, T = the average time in days required by these larvae to reach the 4th instar) has again been used. Under optimum conditions, at 28°C., $N \times \frac{1}{T} = 100 \times \frac{1}{4} = 25$.

Preparation of the Growth Factors

The preparation of the flavin-purine complex has been detailed in a previous paper (2).

The Barium Precipitate and the Barium Filtrate.—The charcoal elute already described (3) was concentrated to a small volume (1 ml. = 100 gm. of liver) and was

* Y. S. acknowledges financial aid from the Markle Foundation.

precipitated with 10 volumes of 95 per cent alcohol and 10 volumes of ether. The mixture was left in the cold room overnight. It was filtered. The filtrate was concentrated to remove alcohol and ether and diluted with distilled water so that 10 ml. = 100 gm. of liver. Then it was extracted 4 times with an equal volume of butyl alcohol.

The butyl alcoholic extracts were combined and concentrated *in vacuo* to dryness. The residue was taken up in 95 per cent alcohol (5 ml. per 100 gm. of liver). The alcohol extract was filtered. The filtrate was neutralized with alcoholic solution of Ba $(OH)_2$. The precipitate was filtered. The precipitate was termed B.P.S. and the filtrate was termed B.F.

Before use, B.P.S. was suspended in water, treated with sulfuric acid to precipitate the barium, and filtered. The pH of the filtrate was adjusted to 5.8-6.0.

Fractions Rich in Pantothenic Acid.—Fraction 355 was the same fraction the preparation of which was described under the number S-13 in a paper dealing with the growth requirements of rats (4).

Fraction 362-6 was a zinc salt. Starting with fraction 355, the ether extract was neutralized with ZnO and concentrated to dryness. The alcohol insoluble portion of the precipitate was discarded. The alcohol soluble portion was concentrated to a small volume and was precipitated with 50-60 volumes of acetone. The precipitate was filtered and dried. The precipitate was found to contain about 20 per cent zinc, 20–30 per cent nicotinic acid amide, 10–15 per cent uracil. This material, before use, was decomposed with H_2S, the sulfide filtered off, and the pH of the filtrate adjusted to 5.8–6.0.

Starting with 362-6, a barium salt of pantothenic acid was prepared. The zinc from 10 gm. of fraction 362-6 was removed by H_2S. Part of the nicotinic acid amide and uracil were removed by adsorption on English fullers' earth. Part of the inert organic acid in the filtrate was removed by the use of the thallium salt. The filtrate was decomposed with HCl, neutralized with Ba $(OH)_2$, and precipitated with acetone. The pantothenic acid was regenerated by the addition of enough $\frac{N}{1}$ sulfuric acid to precipitate the barium. The barium sulfate was filtered off and the pH of the filtrate adjusted to 5.8–6.0.

The pantothenic acid content of the three preparations was determined by their effect on the NY 5 streptococcus, which requires 1 γ of pantothenic acid for maximum growth (5).

RESULTS

Effects of the Barium Precipitate and Barium Filtrate Fractions of Liver Extract

In the presence of killed yeast and flavin-purine complex or riboflavin, the barium precipitate alone supported very little growth of the mosquito larvae. The barium filtrate fraction, at concentrations down to such that 100 ml. of solution contained the material derived from 40 gm. of liver, gave good growth and the addition of B.P.S. had but little effect. At lower concentrations of B.F. the growth fell off markedly and a clear effect of

the further addition of B.P.S. could be demonstrated (Table I). Since the barium precipitate had to be supplied at a concentration such that 100 ml. contained the amount from 150 gm. of liver, it is quite possible that this fraction was merely furnishing more of the same essential substances furnished by the barium filtrate.

Effects similar to that of B.P.S. could be obtained with yeast nucleic acid, at concentrations down to about 20 mg. per 100 ml. of medium, and

TABLE I

Effects of the Barium Filtrate and Barium Precipitate Fractions of Liver Extract, and of Yeast Nucleic Acid and Glutathione in the Presence of the Former Fraction

Concentration						$N \times \frac{1}{T}$
As gm. of liver from which was derived amount of fraction present in 100 ml. of culture fluid			Mg. per 100 ml. culture fluid			
Flavin complex	Barium filtrate	Barium precipitate	Yeast nucleic acid	Glutathione	Riboflavin	
50*	20	0	0	0	0	8.3
50	20	150	0	0	0	19.3
50	0	150	0	0	0	5.4
50	20	0	20	0	0	16.2
50	20	0	10	0	0	13.8
50	25	0	0	0	0	11.2
50	25	150	0	0	0	15.0
50	25	0	33	0	0	17.6
50	25	0	27	0	0	16.7
0	30	0	0	0	0.08	2.1
0	30	0	0	13	0.08	17.6
0	30	0	0	6.7	0.08	11.5
0	30	0	0	3.3	0.08	8.1
0	30	0	10	0	0.08	13.8
0	30	0	10	13	0.08	13.8

* Corresponds to 10 mg. per 100 ml. of culture medium.

with glutathione at a concentration of 13 mg. per 100ml. (Table I). However, the addition of yeast nucleic acid and glutathione together gave no better growth than either substance alone.

Several partially purified fractions from liver extract could also replace the barium precipitate fraction. However, since the barium filtrate fraction alone seemed to contain the major part of the essential growth factors, attention was concentrated on trying to replace it by combinations of the various pure substances isolated from it. A great many experiments were performed, in all of which B.P.S. was supplied at a concentration such that 100 ml. of medium contained the material derived from 150 gm. of liver.

The results were extremely variable and in no case was satisfactory growth obtained. A general impression was gained that β-alanine, adenosine, and nicotinic amide[1], but especially β-alanine, had a slight favorable effect. While tubes containing as supplement only the barium precipitate gave $N \times \frac{1}{T}$ values of 5 or less, those containing B.P.S. together with β-alanine, adenosine, and nicotinic amide frequently gave $N \times \frac{1}{T}$ values over 5 and in one case as high as 12. It became obvious, nevertheless, that further experimentation with the known substances isolated from the barium filtrate would be futile.

Pantothenic Acid and Vitamin B_6

Since the liver preparations containing the mosquito growth factors also contained vitamin B_6 (6), some tests of this substance were performed. It was found that in the presence of killed yeast, flavin-purine complex, and barium precipitate fraction, both crystalline "Factor 1"[1] (7) and crystalline vitamin B_6[1] (8, 9) had a slight but definite growth-promoting effect. Nothing even approaching optimum growth was obtained.

Progress in the purification of pantothenic acid (10, 11), its recognition as one of the factors essential for the growth of chicks (12, 13) and rats (14, 15), and its presence in liver preparations (4) active as mosquito growth factors, led to trials of its effect on the growth of *Aedes aegypti* larvae. Three preparations, two (355 and 362-6) containing about 20 per cent pantothenic acid and one, a barium salt, 60 per cent pantothenic acid and 20 per cent barium, all markedly promoted growth of the larvae. When added to killed yeast and flavin-purine complex, the regenerated zinc salt alone gave fairly high values of $N \times \frac{1}{T}$ and these could be brought almost to the maximum (22.2) by the further addition of vitamin B_6. The optimum concentration of zinc salt in either case was about 0.02 per cent, representing a pantothenic acid concentration of about 4 mg. per 100 ml. The other crude pantothenic acid preparation (355) supported very little growth unless supplemented with vitamin B_6. In the presence of the latter, $N \times \frac{1}{T}$ values of 15 to 20 were obtained when that amount of pantothenic acid was present in 100 ml. which was derived from 100 gm. of liver.

[1] We are grateful to Dr. H. Adkins of the University of Wisconsin for sending us some nicotinic amide, to Dr. S. Lepkovsky for a sample of his crystalline Factor 1, and to Merck and Co. for supplies of vitamin B_6-hydrochloride.

The most significant results were obtained with pantothenic acid regenerated from its barium salt (Table II). In the presence of killed yeast, flavin-purine complex, vitamin B_6, and glutathione, growth was very slight and no larvae reached the 4th instar. The further addition of the regenerated barium salt of pantothenic acid enabled growth to proceed to the adult stage. As is evident from Table II, a concentration of 6.7 mg. of barium salt per 100 ml., corresponding to about 4 mg. of pantothenic acid per 100 ml., gave the best growth. The value of $N \times \frac{1}{T}$ was 15.8 in one experiment and 21.4 in another, and almost all of the larvae reached the adult stage in 13 to 14 days. If glutathione was omitted, growth was always not quite as rapid as in its presence and fewer of the larvae reached the adult stage. If B_6 was omitted growth was very slow and few or none of the larvae reached the 4th instar (Table II). If riboflavin was substituted for the flavin-purine complex, growth proceeded at a much slower rate (Table II). A few of the larvae nevertheless reached the adult stage in this medium in which the liver extract was replaced entirely by known substances.

TABLE II

The Effects of Pantothenic Acid and Vitamin B_6

Concentration mg. per 100 ml. culture fluid						$N \times \frac{1}{T}$	Adults from 6 larvae		Average days to reach adult stage
Flavin complex	Riboflavin	B_6	Regenerated barium pantothenate*	Glutathione	Nicotinic amide		♀	♂	
10	0	0	6.7 (4)	0	0	1.9	0	0	
10	0	1.3	6.7 (4)	0	0	13.8	1	2	12
10	0	0	6.7 (4)	13	0	0	0	0	
10	0	1.3	6.7 (4)	13	0	15.8	4	1	14.5
10	0	1.3	6.7 (4)	13	8	17.6	2	4	14.5
0	0.04	0	6.7 (4)	0	0	0	0	0	
0	0.04	1.3	6.7 (4)	0	0	11.2	1	0	22
10	0	1.3	0	13	0	0	0	0	
10	0	1.3	13.3 (8)	13	0	9.2	1	2	17.5
10	0	1.3	6.7 (4)	13	0	21.4	2	4	13.5
10	0	1.3	3.3 (2)	13	0	9.6	1	2	18

* Number in parentheses gives the approximate concentration of pantothenic acid.

The addition to a medium of killed yeast, flavin-purine complex, vitamin B_6, pantothenic acid, and glutathione, of various other substances such as yeast nucleic acid, β-alanine, adenosine, tryptophane-betaine, nicotinic amide, inosine, etc., had no effect except in the case of nicotinic amide which in some experiments slightly accelerated growth (Table II).

Vitamin B_6, which has been recently synthesized and shown to be 2-methyl-3-hydroxy-4,5-di-(hydroxymethyl) pyridine (16), was absolutely essential for growth (Table II). Whether a more or less nearly pure preparation of pantothenic acid was used, *the optimal concentration of vitamin B_6 was 1.3 mg. per 100 ml. of medium.*

DISCUSSION

The major accessory factors, essential for the growth of mosquito larvae and supplied by liver extract in a medium of killed yeast in liver extract, appear to be riboflavin, pantothenic acid, and vitamin B_6, with glutathione and nicotinic amide having a lesser growth-stimulating rôle. It may be that the killed yeast, which supplies enough thiamin, also supplies almost enough glutathione and nicotinic amide. The experimental results show clearly that other essential substances supplied by liver extract remain to be discovered. These are present in the barium filtrate and more especially in the flavin-purine complex. While killed yeast, vitamin B_6, pantothenic acid, and glutathione, with flavin-purine complex, gave excellent growth, the same materials with pure riboflavin gave considerably slower growth. The rôle of yeast nucleic acid is difficult to evaluate. Although it could be substituted for the barium precipitate fraction in the presence of the barium filtrate, it had no effect on the growth obtained in the presence of flavin complex or riboflavin plus vitamin B_6, pantothenic acid, and glutathione.

At present, one can safely conclude that *Aedes aegypti* larvae require, as accessory growth factors, thiamin, riboflavin, pantothenic acid, and vitamin B_6; probably glutathione and nicotinic amide, and certainly other as yet unknown substances present in yeast and in liver extract. Since pantothenic acid consists of β-alanine in amide linkage with a hydroxy acid (11), one may account for the observed slight favorable effects of β-alanine by assuming that some individual larvae have very limited powers of synthesizing pantothenic acid if they are supplied with β-alanine, as is the case with certain strains of bacteria (23, 24).

Older work on the growth factor requirements of insects has been previously noted (2). The flies *Lucilia sericata* (17) and *Drosophila melanogaster* (18) and the beetle *Dermestes volpinus* (19) require cholesterol. Both *L. sericata* (20) and *D. melanogaster* (21) require thiamin, and the latter also requires riboflavin (21). Tatum (22) has recently found that *Drosophila* larvae required, in the presence of all the known vitamins, three additional factors present in yeast autolysate. Two of these could be separated from each other by precipitation with barium hydroxide in alcoholic solution,

a separation which suggests that they may be the same as the barium precipitate and filtrate fractions required by *Aedes aegypti* and that one of them may be pantothenic acid. Tatum (22) also showed that nicotinic acid is essential for the growth of *Drosophila* larvae.

As more work is done, it becomes increasingly obvious that insects require the same growth factors of the vitamin B complex group as do vertebrates. This is not surprising, since numerous species and strains of bacteria and yeast also require the same substances for growth (5, 23–26). Other species and strains of bacteria and other microorganisms can synthesize one or more of these factors (23, 24, 27). Microorganisms of this latter group under natural conditions supply mosquito larvae with their essential growth factors.

SUMMARY

The larvae of *Aedes aegypti* grew normally under sterile conditions in a medium consisting of killed yeast, flavin complex, or riboflavin, and two fractions derived from liver extract and designated as the barium filtrate and barium precipitate. The latter fraction could be replaced by yeast nucleic acid or by glutathione.

The larvae also grew at an almost optimal rate in a medium consisting of killed yeast, flavin-purine complex, vitamin B_6, pantothenic acid, and glutathione. All of these constituents except the glutathione were absolutely essential. Replacement of the flavin-purine complex by pure riboflavin resulted in slower growth, but nevertheless some larvae reached the adult stage.

REFERENCES

1. Trager, W., *Am. J. Hyg.*, 1935, **22**, 18.
2. Trager, W., and SubbaRow, Y., *Biol. Bull.*, 1938, **75**, 75.
3. SubbaRow, Y., Jacobson, B. M., and Fiske, C. H., *New England J. Med.*, 1936, **214**, 194.
4. Hitchings, G. H., and SubbaRow, Y., *J. Nutrition*, 1939, **18**, 265.
5. SubbaRow, Y., and Rane, L., *J. Am. Chem. Soc.*, 1939, **61**, 1616.
6. Dann, N. J., and SubbaRow, Y., *J. Nutrition*, 1938, **16**, 183.
7. Lepkovsky, S., *J. Biol. Chem.*, 1938, **124**, 125.
8. György, P., *J. Am. Chem. Soc.*, 1938, **60**, 983.
9. Keresztesy, J. C., and Stevens, J. R., *Proc. Soc. Exp. Biol. and Med.*, 1938, **38**, 64.
10. Williams, R. J., Truesdail, J. H., Weinstock, H. H., Rohrmann, E., Lyman, C. M., and McBurney, C. H., *J. Am. Chem. Soc.*, 1938, **60**, 2719.
11. Williams, R. J., Weinstock, H. H., Rohrmann, E., Truesdail, J. H., Mitchell, H. K., and Meyer, C. E., *J. Am. Chem. Soc.*, 1939, **61**, 454.
12. Woolley, D. W., Waisman, H. A., and Elvehjem, C. A., *J. Am. Chem. Soc.*, 1939, **61**, 977.

13. Jukes, T. H., *J. Biol. Chem.*, 1939, **129**, 225.
14. SubbaRow, Y., and Hitchings, G. H., *J. Am. Chem. Soc.*, 1939, **61**, 1615.
15. Oleson, J. J., Woolley, D. W., and Elvehjem, C. A., *Proc. Soc. Exp. Biol. and Med.*, 1939, **42**, 151.
16. Harris, S. A., and Folkers, K., *J. Am. Chem. Soc.*, 1939, **61**, 1245.
17. Hobson, R. P., *Biochem. J.*, London, 1935, **29**, 2023.
18. Van't Hoog, E. G., *Z. Vitaminforschung*, 1936, **5**, 118.
19. Gay, F. J., *J. Exp. Zool.*, 1938, **79**, 93.
20. Hobson, R. P., *Biochem. J.*, London, 1933, **27**, 1899.
21. Van't Hoog, E. G., *Z. Vitaminforschung*, 1935, **4**, 300.
22. Tatum, E. L., *Proc. Nat. Acad. Sc.*, 1939, **25**, 490.
23. Evans, W. C., Handley, W. R. C., and Happold, F. C., *Brit. J. Exp. Path.*, 1939, **20**, 396.
24. Mueller, J. H., and Klotz, A. W., *J. Am. Chem. Soc.*, 1938, **60**, 3086.
25. Snell, E. E., Strong, F. M., and Peterson, W. H., *J. Am. Chem. Soc.*, 1938, **60**, 2825.
26. Schultz, A. S., Atkin, L., and Frey, C. N., *J. Am. Chem. Soc.*, 1939, **61**, 1931.
27. Knight, B. C. J. G., Bacterial nutrition, *Great Britain Med. Research Council, Special Rep. Series*, No. 210, 1936, 182 pp.

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A DWARF MUTATION IN THE RABBIT

THE CONSTITUTIONAL INFLUENCE ON HOMOZYGOUS AND HETEROZYGOUS INDIVIDUALS

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PLATES 48 TO 50

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Size, physical conformation and character of coat are the chief distinguishing marks of standard races of rabbits. All of these characters are inherited and by selective breeding have been utilized in the development and perfection of numerous breeds. The factor of size is of particular interest as an expression of inherent racial attributes and there exists an eight- to tenfold difference in the adult weights of individuals of the largest and smallest racial groups. The smallest breed is the Polish which is said to have been developed by selective breeding of diminutive forms occurring among other breeds, particularly the Dutch. Hence, the Polish rabbit occupies a unique position. It is not only the smallest of standard breeds but is also a dwarf form.

In the course of our studies on constitution, three strains of Polish rabbits have been obtained from widely separated sources. Tests showed that all of these were of Dutch origin. They were also of Dutch conformation and, except for the fact that they were albinos, were in reality diminutive Dutch rabbits. However, they varied considerably in size and it was found that the smaller individuals of two of these strains transmitted a still smaller lethal type of dwarf entirely different from the normal young of Polish rabbits.

A brief note on this condition was published in 1934 (1). It is one of several variations characterized by a diminutive or dwarf-like form, present at birth, with or without other distinguishing characteristics, which have been encountered in the course of an extensive investigation of hereditary constitutional variations in an animal population. Taken as a whole, this group of diminutive conditions represents a class of variations which is of frequent occurrence in the rabbit and includes forms which are comparable with the so called runts of domestic animals. There are, however, funda-

mental differences between the various types comprising the group. There are, on the one hand, diminutive forms which appear to be attributable to size variations of the same genetic order as those which characterize the size variations of standard breeds and have produced animals of the Polish type (2). In addition, it has been found that location in the uterus has a distinct effect on birth weights and that, in general, size increases with the distance from the uterine os (3). But there are other diminutive forms which are definitely pathological and, while the expression of these conditions is undoubtedly subject to the influence of such factors as those mentioned, they are variations of a different order and are due primarily to other causes. The determination of the genetic and constitutional significance of the several diminutive forms is, therefore, a problem of considerable importance from a functional as well as a morphological point of view.

For this reason, an extensive investigation of the lethal dwarf mutation was undertaken. In the course of this investigation, it became apparent that the mutation which rendered homozygous individuals incapable of living exercised a profound influence on heterozygous individuals as well and that some of the most important effects of this gene did not become apparent until maturity or later in life. Moreover, it was found that the effect of the dwarf gene on the adult as well as on the newborn animal was greatly modified by the association with other genetic groups which were introduced intentionally or by chance in the course of routine hybrid matings.

The results to be reported are, therefore, divisible into three categories: namely, those relating to the action of the dwarf gene as indicated by conditions present at birth and the mode of inheritance of the dwarf mutation; second, the immediate effects of various crosses on the expression of the dwarf gene; and third, the status of adult animals, including heterozygous dwarf transmitters and other progeny derived from the crossing of the original dwarf line with unrelated animals from presumably normal and abnormal lines. The first two categories will be covered in the present paper and reference will be made to the status of adult animals, but a more complete description of this phase of the study will be reported in a later paper.

Materials and Methods

The first instance of the dwarf variation to be described in this paper was found in 1931 in a litter derived from a backcross mating of a Polish hybrid female. Subsequent investigation showed that the transmission of the variation was limited to one family of our pure bred Polish stock and to hybrids derived from that family.

The abnormality is invariably lethal in homozygous form and genetic studies were necessarily carried out with heterozygous animals. Progeny obtained by mating Polish transmitters with unrelated animals were tested and those found to transmit the variation were interbred to form an F_2 generation in which the proportion of normal and abnormal individuals was determined.

Progeny derived from the outcross mating of Polish transmitters were marked for future identification and weighed as soon as possible after birth on a Toledo automatic balance calibrated in gram intervals. The body weights of members of the F_2 generation were obtained in a similar manner and representatives of the various weight classes were killed for organ weight determinations and histological study. The organs of dwarfs were weighed on a chemical balance, while those of larger animals were weighed on a torsion balance calibrated in milligram intervals.

A series of experiments of this nature was carried out using animals derived from crosses between Polish dwarf transmitters and members of various pure bred and hybrid lines, some of which were known to transmit functional abnormalities. Dwarfs and transmitters obtained in this manner were studied to determine whether or not the new factors changed the variation from the original form observed in the Polish.

Numerous attempts were made to raise dwarfs by artificial feeding and by the administration of various hormones. In addition, members of the other F_2 classes were held under observation to study their reaction to ordinary environmental conditions and to determine their ultimate fate.

Routine tissues for microscopic examination were fixed in Petrunkewitsch's solution and stained with hematoxylin and eosin. Pituitary glands were fixed in Susa's solution and stained with a modification of Mallory's aniline blue method.

RESULTS

Numerous pure bred and hybrid lines have been tested, but aside from minor influences on viability of questionable significance the only line found to carry factors which modify the expression of the dwarf variation is one that carries a cretinoid abnormality (4). The factors carried by this line modify the body weights of transmitters and dwarfs but have no apparent effect on the proportion of affected individuals in different genetic generations.

Physical Appearance

A pronounced reduction in size is the most obvious physical alteration and dwarfs are approximately one-third the size of their normal litter mates (Fig. 1). In addition, an abnormal configuration of the head distinguishes the animals and serves to differentiate affected individuals from diminutive forms due to other causes (Fig. 2).

The posterior calvarium is rounded and bombose but its slope breaks abruptly in the supra-orbital region, giving the snout a characteristic dished-out appearance. The ears are small and because of the enlarged calvarium appear to be set abnormally far back in the head. This appear-

ance is frequently so marked as to suggest hydrocephalus but examination shows no increase in fluid. The frontal and parietal bones are calcified only at their inferior borders and throughout the remainder of their extent are represented solely by membrane.

Rarely, a second abnormal dwarf type occurs in the litters of dwarf transmitters. These animals are slightly larger than dwarfs and the bones of the calvarium are almost completely calcified at birth. In subsequent paragraphs animals of this type will be referred to as semi-dwarfs.

As a general rule, both dwarfs and semi-dwarfs are well nourished at birth and possess abundant subcutaneous fat deposits. With the exception *of the abnormal skull shape, their bodily proportions are uniformly reduced and they appear as miniature reproductions of their normal litter mates. In rare instances (0.3 per cent),* dwarfs derived from crosses with the *cretinoid line show other alterations.* Such animals are always born dead and present general *myxedema* of the skin with chyliform effusions in the peritoneum, pleura and pericardium.

Inheritance

More than 1,000 progeny have been obtained from the crossing of dwarf transmitters and normal unrelated animals but only 2 dwarfs have occurred in this generation. These were both found in consecutive litters of the same parents, a Himalayan female and a dwarf transmitting male. Similar matings involving other related Himalayan females were undertaken in view of the possibility that this family carried dwarfing factors of the same nature, but no dwarfs were obtained. It should be noted, however, that an abnormality expressed as an undersized, dwarfish, "ratty" individual is occasionally found in litters produced by pure bred matings within this family and it is not impossible that a concentration of the factors concerned in this abnormality in combination with the dwarf gene from the Polish line may have produced the variation in question. In any case, it is certain that the occurrence of dwarfs in this instance was due to a peculiarity of the normal parent rather than to the dominance of the dwarf gene.

At the present time, the interbreeding of dwarf transmitters has given rise to 1,321 progeny of which 305 were dwarfs and 33 semi-dwarfs. On the basis of a simple recessive unit factor, 25 per cent or 330 of the animals would be expected to show the dwarf variation. Actually, the variation appeared in 23 per cent of the progeny and the difference between this figure and the expected value is not significant from a genetic point of view.

The exact status of semi-dwarfs is open to question. Relatively few of

these animals have been reared to maturity and breeding tests have not been numerous, but the results obtained are highly suggestive and of sufficient interest to warrant some consideration.

To date, the interbreeding of semi-dwarfs has given rise to 23 progeny of which 6 were normal in appearance, 11 were semi-dwarfs and 6 were dwarfs—a close approximation to a 1:2:1 ratio. On the other hand, the breeding of these animals with ordinary dwarf transmitters resulted in 30 progeny of which 16 were normal, 6 were semi-dwarfs, and 8 were dwarfs—an approximate 2:1:1 proportion. In each case, there occurred the same 3 to 1 ratio of dwarfs that was observed in the interbreeding of ordinary dwarf transmitters and it is apparent that the two classes of animals were not different with respect to the dwarfing factor.

However, the nature of the differentiating factor is indicated by the occasional occurrence of an hereditary size variation leading to a diminutive individual in non-dwarf transmitting Polish stock. It is suggested that such a variation was incorporated by chance in the dwarf producing stock and that the semi-dwarf represents an animal of this class rendered smaller by the action of the dwarf gene. The ability of the dwarf gene to exert such an influence in heterozygous animals will be discussed in a later paragraph and is demonstrated by the size reduction which distinguishes transmitters of the condition.

According to such a conception, semi-dwarfs would be genetically heterozygous for the dwarfing factor and homozygous for the diminutive factor with a genotype of *Aabb* in which *a* represents the dwarf gene and *b* the diminutive gene. Checkerboards representing crosses between semi-dwarfs and between semi-dwarfs and ordinary transmitters are presented in Charts 1 and 2. It will be observed that the theoretical ratios of 1:2:1 in the first instance and 2:1:1 in the second are identical with those obtained in actual breeding experiments.

Birth Weight

Two distinct weight classes of approximately equal size are found in the litters of Polish dwarf transmitters when mated with normal unrelated animals of any line other than that which carries the cretinoid abnormality. Members of the heavier class do not produce the variation and progeny derived from interbreeding are of a uniform size. On the other hand, representatives of the smaller class transmit the dwarf variation when interbred and, in addition, their litters contain the abnormal weight class referred to above as well as normal sized individuals (Fig. 1).

At birth, the weights of dwarfs vary between 11 and 23 gm. with an average of 15 gm., while semi-dwarfs weigh from 22 to 33 gm. with an average of 26 gm. A determination of the relationship of the various weight classes in individual litters shows that the weights of dwarfs average 35.4

	Ab	*ab*
Ab	*AAbb* Normal	*Aabb* Semi-dwarf
ab	*Aabb* Semi-dwarf	*aabb* Dwarf

CHART 1. Checkerboard showing the expected composition of the generation obtained by crossing semi-dwarfs of a genotype *Aabb*.

	AB	*Ab*	*aB*	*ab*
Ab	*AABb* Normal	*AAbb* Normal	*AaBb* Normal	*Aabb* Semi-dwarf
ab	*AaBb* Normal	*Aabb* Semi-dwarf	*aaBb* Dwarf	*aabb* Dwarf

CHART 2. Checkerboard showing the expected composition of the generation obtained by crossing semi-dwarfs (*Aabb*) and ordinary dwarf transmitters (*AaBb*).

per cent of the weight of their heaviest litter mate, and by the same standard, the weights of semi-dwarfs average 45.5 per cent and transmitters 74.7 per cent. In contrast, the weights of normal members of these litters average 94 per cent of the weight of the heaviest sib.

The birth weights of animals derived from crossing Polish dwarf transmitters with normal, unrelated rabbits and from the interbreeding of transmitters obtained in this manner are plotted in Chart 3 to show the frequency

with which the different weight classes occurred. The curve representing the first generation approaches a normal frequency curve while that of the second generation is bimodal. The spread of the first phase of the bimodal curve corresponds with that of the first generation but encompasses roughly only 75 per cent of the area showing that, while the second generation contained the same weight classes as were present in the first generation, only

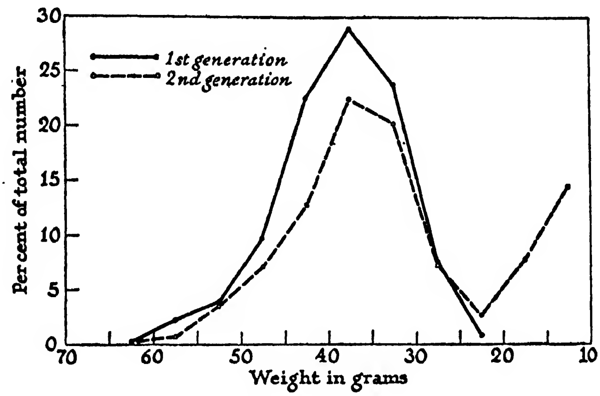


CHART 3

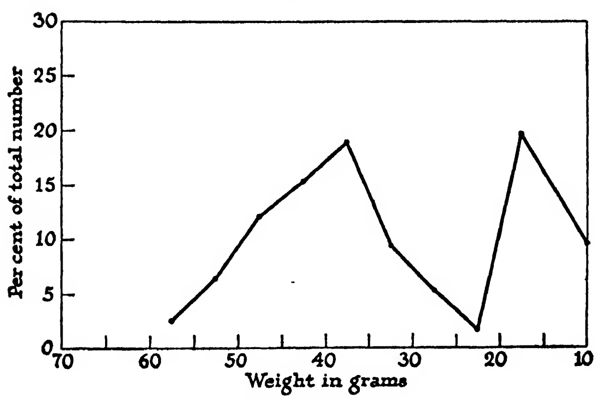


CHART 4

about 75 per cent of the animals fell into those classes. The second phase of the bimodal curve represents dwarfs and covers only one-quarter of the total area as would be expected if the factor determining these variations was simple and recessive in nature.

A clear-cut birth weight distinction between transmitters and non-transmitters does not occur in lines which carry the cretinoid abnormality. In many instances, the smaller members of a litter prove to be transmitters but, on the other hand, transmitters often weigh as much or more than

non-transmitters. In the group as a whole the birth weight of transmitters averaged 85 per cent of that of the heaviest litter mate, which is approximately 10 per cent higher than that observed in other transmitters. In addition, the dwarfs obtained from this line are generally heavier and their weight averages 40.6 per cent of that of the largest litter mate in contrast to 35.4 per cent observed in the case of other dwarfs. The birth weights of progeny obtained from the interbreeding of transmitters of this type are plotted in Chart 4 and a comparison with the second generation curve presented in Chart 3 brings out the differences mentioned.

Viability and Eventual Fate

The raising of transmitters of the dwarf variation presents no great difficulty. Semi-dwarfs are viable but special care is usually required during the first month of life. On the other hand, while more than 300 dwarfs have been studied, survival for more than 5 days has only been observed in instances in which the expression of the variation was modified by the introduction of factors carried by the cretinoid line.

As a rule, the dwarfs are born alive but die within 48 hours. They are capable of nursing and will usually gorge themselves at the first feeding. Occasionally they will nurse a second or third time but, thereafter, although their efforts may continue vigorous, they generally fail to obtain milk. They may nurse successfully for a few additional feedings if held to the doe's breast but, in a short time, this procedure fails and the animals cease to suckle or are unable to obtain milk. The feeding of cow's milk or of material obtained from the stomachs of killed nurselings may prolong life for a few days but eventually, despite a full stomach, the animals die.

The administration of thyroid extracts at birth or to the mother during the last days of gestation has been without appreciable effect in prolonging life. In like manner, treatment with the growth hormone of the pituitary has been unsuccessful. On the other hand, the implantation of whole pituitary glands obtained from normal litter mates into the subcutaneous tissues of dwarfs maintained life for as long as 5 days in a number of cases.

Therapeutic measures were not attempted in the 4 instances in which life continued for more than 5 days and the animals were left undisturbed in their mother's care. It should be emphasized, however, that in all of these cases one or both parents carried factors concerned in the production of the cretinoid abnormality. 2 of the dwarfs were litter mates but the remaining animals were nursed by different mothers and were born at different periods of the year.

One dwarf weighed 17 gm. at birth, survived for 14 days and died weighing 21 gm. A second dwarf weighed 23 gm. at birth, survived 23 days and at death weighed 44 gm. In a third case the birth weight was 22 gm., the animal lived 26 days and died weighing 68 gm., and in a fourth instance the birth weight was 19 gm., the survival period 55 days and the death weight 96 gm. In the 3 latter cases, the growth rate paralleled that of normal animals for approximately three-quarters of the survival period but then reached a plateau and the animals died immediately after a loss of weight became evident.

The appearance of survivors was characteristic and, if the animals had been found in the wild at the end of a month of life, their species identification might be in doubt (Figs. 3, 4 and 5). The bones of the skull became calcified but the bombose configuration of the calvarium persisted. The ears remained small and resembled those of a kitten. The abdomen was full and rounded and there were large deposits of fat in the region of the shoulder girdle. The hair was soft and silky. During the period of active growth, the animals were vigorous and playful but with cessation of growth they became apathetic and sat hunched in one position throughout the day. Usually, diarrhea was present during the last days of life.

The survival periods of semi-dwarfs and of transmitters are indefinite and animals of the latter class have been held for as long as 5 years. The growth rate of transmitters is comparable with that of normal animals but the size alteration observed at birth persists throughout life and the variation in adult weight is of a similar order. In the case of semi-dwarfs, growth persists for a longer period of time so that their adult weight approaches that of ordinary transmitters and birth weight relationships do not obtain after maturity.

The great majority of transmitters present no external physical alteration other than that of size but abnormalities worthy of comment have occurred in several instances. Cataracts of the lens and structural variations of the iris appear with considerable frequency in first generation hybrids derived from the Himalayan breed and recur in subsequent generations. On the other hand, such abnormalities as kyphosis of the spine and internal bowing of the forelegs which occasionally appear in F_1 hybrids apparently are not transmitted to a second generation. An unusual variation in the calvarium consisting of symmetrically placed defects in the frontal bones has frequently been observed at autopsy (Fig. 6). The defects occasionally persist throughout life as sharply circumscribed oval areas completely devoid of bone. In other cases, healing occurs but a persistent fissure marks the site of the defect. The variation occurs with the highest incidence in transmitters derived from the Himalayan breed but also occurs in transmitters obtained from other breeds and is occasionally seen in non-transmitters.

There is no evidence to suggest that any of the abnormalities enumerated form an integral part of the dwarf variation. It would appear, on the other hand, that they are independent variations and that their occurrence in dwarf transmitters is due to chance association.

The early history of the animals is usually uneventful and the majority are no more subject to the disorders of immaturity than their normal litter mates. Some animals, however, particularly those derived from a Himalayan cross, present a disorder characterized by the resorption of calcium which may lead to spontaneous fracture or bending of the long bones. Death may occur after a period distinguished by loss of appetite, arrested growth and general debility and is usually preceded by signs of an acute gastro-intestinal upset. Recovery is the rule but such animals are subject to further disturbances of a similar order after maturity is attained.

The majority of female semi-dwarfs and transmitters become overfat at maturity and, unless they are bred at frequent intervals, the fat in normal regions accumulates to an abnormal extent. Large deposits of fat are also found in abnormal depots, particularly in the anterior triangle of the neck and about the shoulder girdle. Despite this manifestation the animals remain alert and active without the apathetic disposition which usually characterizes adipose animals. Males do not become overfat but continue vigorous and thrifty and are frequently characterized by a pugnacious disposition.

The fertility of both males and females is high but litter size is generally smaller than normal and averages 3 to 4, rather than 5 to 6 as in the general rabbit population. The susceptibility to snuffles, a common upper respiratory infection in the rabbit, is intermediate and the susceptibility to gastro-intestinal disturbances is below the average.

A recurrence of the disturbance of calcium metabolism observed in early life is common in recovered animals but the disorder also occurs in animals whose early development proceeded in a normal manner. Manifestations of the disorder are particularly apparent in the calvarium and teeth. The bones of the calvarium, especially the parietal bones, are roughened and at autopsy are found covered with deep erosions. The incisor teeth are pitted and frequently are ground down to the gum margin. The molar teeth are worn into irregular shapes with sharp edges which produce chronic ulcers on the tongue and cheek. Occasionally, the crowns are flattened and the teeth bent inward to meet in the midline, forming a bony arch over the tongue which may be almost completely bisected by ulceration. Such animals are unable to masticate and would die of starvation if the condition went undetected.

Females are particularly susceptible to toxemia of pregnancy in their third or fourth gestation (5, 6). The incidence of deaths due to this disorder is 30 per cent in contrast to an incidence of 19 per cent in non-transmitters of the same derivation. Animals that survive the disorders of calcium metabolism and toxemia of pregnancy for 4 years almost invariably show adenomata or adenocarcinomata of the uterine fundus. This finding was noted in a previous report (7) and will be considered in detail in a later paper. Males are generally distinguished by a long productive breeding history. There has been no evidence that they were susceptible to endocrine disturbances in later life and the incidence of tumors is no greater than in the general male population of the colony.

Postmortem Findings

At birth dwarfs show no gross abnormality other than a reduction in size. Animals that survive to the 4th or 5th day frequently show a unilateral or bilateral hydronephrosis and, in such cases, the ureters are imperfectly developed with either a lumen or an attachment to the bladder lacking. In other instances, no organic lesion is found, the stomach contains milk and there are abundant deposits of pinkish fat throughout the body.

Pneumonia was the immediate cause of death of the dwarf that survived for 14 days, while the 2 animals that lived for 23 and 26 days respectively showed no gross lesion to account for death other than a moderate bilateral hydronephrosis. Atrophy of the gonads was a striking feature in these animals. One was a male and 2 were females but the gonads were only found after a thorough search and their identity as ovaries or testicles could not be determined by gross examination. The single animal that lived for 55 days showed small hemorrhages on the surface of the intestine but the pelvis of the kidney was not dilated and the ureters were patent. No gonads were found but examination of the external genitalia suggested that the animal was a male.

Organ Weights

The weights of the organs of dwarfs, transmitters and their normal litter mates were determined at birth and the averaged figures are presented in Table I. Both actual weights and percentage values computed from the net body weight are given. The net body weight was obtained by subtracting the weight of the gastro-intestinal mass from the gross body weight.

The differentiation of normal and transmitting animals at birth was based on gross body weight. The weight limits of these classes were

determined by rearing and testing numerous progeny, and to compensate for the possibility of overlap and to minimize the danger of erroneous classification the animals used for birth organ weight determinations were selected from the middle of their respective groups. Unfortunately, the organ weights of newborn semi-dwarfs are not available. These animals are comparatively rare and it seemed more advantageous to raise them for genetic studies than to kill them for this purpose.

TABLE I

The Averaged Organ Weights of Normal Animals, Dwarf Transmitters and Dwarfs Obtained at Birth

Classification	Net body weight	Thyroid		Pituitary		Left suprarenal		Thymus		Spleen	
		Actual weight	Per cent of net body weight								
	gm.	*mg.*	*per cent*	*mg.*	*per cent*	*mg.*	*per cent*	*mg.*	*per cent*	*mg.*	*per cent*
Normal..........	37.5	6.80	0.018	2.40	0.006	2.30	0.006	60.7	0.162	11.90	0.031
Transmitter.....	28.9	5.05	0.017	1.23	0.004	1.37	0.004	47.3	0.163	5.73	0.019
Dwarf..........	15.9	1.90	0.012	0.45	0.002	0.70	0.004	31.5	0.196	2.60	0.019

Classification	Liver		Left kidney		Heart		Lungs		Brain	
	mg.	*per cent*	*mg.*	*per cent*	*mg.*	*per cent*	*mg.*	*per cent*	*mg.*	*per cent*
Normal.........	2474.5	6.59	187.0	0.490	250.7	0.668	564	1.50	1223.5	3.25
Transmitter....	1796.3	6.21	153.3	0.523	199.0	0.668	457	1.58	1145.3	3.96
Dwarf.........	947.0	5.91	97.0	0.606	128.0	0.800	258	1.61	949.0	5.93

It should be noted that an estimation of the weight of the thyroid gland in young animals is extremely difficult. The organ is not distinct from surrounding structures and arbitrary limits must be assigned to the amount of tissue to be removed for weighing. It is obvious that the determinations cannot be accepted as actual weights of the thyroid gland but the procedure and the limits of the tissue removed were the same in all instances and for these reasons the determinations are of value as relative measures of the weight of the organ.

The actual weights of all organs were decreased in transmitters and lowest in dwarfs but a comparison based on the relation to net body weight shows that in a number of cases the decrease was not proportionate and that in

others the relative weight was actually increased. The amounts of thyroid, suprarenal, pituitary, liver and spleen substance per gram of net body weight were reduced in transmitters and least in dwarfs, while the relative amounts of thymus, kidney, heart, lung and brain substance were increased in transmitters and largest in dwarfs. The decrease in the relative weight of organs in dwarfs was most pronounced in the case of the thyroid, pituitary and suprarenal while the increase was greatest in the case of the brain.

Microscopic Examination at Birth

The dwarfs show no striking histological abnormality. Individual cells are not reduced in size but are present in decreased numbers.

Both acidophiles and basophiles are found in the pituitary and their proportion is not significantly different from that observed in normal litter mates (Fig. 8). If any alteration exists it lies in the direction of the increased number of granular elements with a corresponding reduction in chromophobes. Sections of the thyroid show a well organized structure of normal appearing follicles filled with deep-staining colloid. The different zones of the suprarenal cortex are not well defined and individual cells are hazy and stain poorly, but the relative amounts of cortex and medulla are comparable with those found in normal newborn animals. The liver and spleen contain an excessive amount of hematopoietic tissue.

Microscopic Examination of Survivors

With the exception of the hypophysis, the organs of the 4 survivors showed few abnormal histological changes other than those associated with immaturity. Numerous foci of hematopoietic tissue persisted in the liver and spleen and the abundance of dark-staining endothelial nuclei which characterize the kidney glomeruli of newborn were still present in the 55 day old animal. The various zones of the suprarenal could not be recognized at any period and the staining properties of the cells became progressively poorer, so that in the older animals cell outlines could only be distinguished with difficulty. The follicles of the thyroid increased in size with age and, in the older animals, no evidence of colloid absorption could be detected.

The striking alteration in the hypophysis was the pronounced increase in the size and number of acidophiles (Figs. 7, 9, 10 and 11). The increase was generalized throughout the anterior lobe and in many areas no other elements could be found. Scattered foci of basophiles were present in other instances and these cells were also greatly enlarged. The intermediate

lobe was narrow and the cleft representing the lumen of Rathke's pouch persisted even in the 55 day old animals.

DISCUSSION

Hereditary forms of dwarfism have been described in man (8), mice (9), guinea pigs (10) and rats (11). Two types of the disorder have been differentiated in man; nannosomia primordialis, in which retarded growth is apparent at birth and, nannosomia infantilis, in which individuals are of normal size at birth but cease to develop during early childhood. The determining factors in the development of the first type are not understood, while the pathogenesis of the second type is associated with achondroplasia, rickets, or abnormalities of the pituitary and thyroid. The dwarfs described in mice, guinea pigs and rats are of the latter class but both forms are found in the rabbit. The present investigations have been concerned entirely with the form evident at birth and a consideration of the results obtained throws some light on its pathogenesis.

The abnormality is hereditary and determined by a simple recessive unit factor but the factor also exerts an influence in heterozygous animals where its action is likewise manifest by a reduction in size. The site of action of the factor is not apparent from an examination of ordinary dwarfs but a study of the few survivors that occurred in crosses with the cretinoid line suggests that the organ primarily affected by the hereditary variation may be the pituitary.

The disproportionate reduction in the weights of the endocrine organs of dwarfs and transmitters at birth indicates a primary fault in this system, but histological examination reveals no striking alteration. The thyroid and suprarenal show little modification in surviving dwarfs but the normal histological elements of the anterior lobe of the pituitary are completely replaced by hypertrophic acidophiles, and this change is associated with atrophy of the gonads. The animals are thus the antithesis of the dwarf mice described by Smith and MacDowell in which acidophiles are absent and the reproductive system highly developed (12). In such instances, there is apparently a suppression of the growth-promoting hormone without a corresponding suppression of the gonadotropic hormone, whereas in the animals under discussion suppression of the gonad-stimulating hormone appears to be an important feature.

Apparently, the factor responsible for the longer survival of dwarfs obtained from crosses involving the cretinoid line results in a greater activity of the acidophilic cells and thus supplies growth-stimulating substances which are absent in ordinary dwarfs. The greater birth weight of

both dwarfs and transmitters of this line may conceivably be due to prenatal activity of this nature. However, the addition of this factor is not sufficient to prolong life beyond a certain limit and death is apparently related to the absence of the gonadotropic hormone or of some genetically related substance. The failure of ordinary dwarfs to survive may in like manner be due to a more complete paralysis of the secretory function of the pituitary. The survival to birth in such cases might be explained by the utilization of maternal hormones.

The surviving dwarfs resemble in some respects the dwarfs produced by Zondek in infantile rats (13). Following continued treatment with estrin he observed a retardation of growth amounting to 43 per cent as judged by controls, together with a complete arrest in gonad development, and advanced the explanation that estrin acted to inhibit the secretion of pituitary hormones. A possible association with an abnormality in estrin metabolism in the present instances is further suggested by the great number of carriers of the dwarf gene that eventually develop adenocarcinoma of the uterine fundus. This association will be considered in detail in a later paper.

In any case, the evidence at hand indicates that the pituitary is so affected by an hereditary variation that its secretory functions are inhibited. In homozygous individuals, the inhibition is complete and the variation is expressed as a lethal dwarf. In heterozygous animals, the function of the organ is altered, producing an undersized individual with a high susceptibility to uterine cancer. The introduction of factors from the cretinoid line either partially removes the inhibition so as to allow hyperplasia and function of the acidophiles or alters the constitution of the animals in such manner that life is possible for a short period without the full component of pituitary hormones. Theoretically, it should be possible to maintain life indefinitely by replacement therapy. The implantation of whole pituitary glands has prolonged life 2 to 3 days beyond the average in a number of cases and its eventual failure may be related either to an insufficient amount of available hormone in the implanted glands or to mechanical difficulties in absorption. Further investigations along this line are in progress.

It is of interest from a genetic point of view that the reduction in size of dwarfs and transmitters is of a measured order. The homozygous lethal dwarf is one-third and the heterozygous transmitting animal two-thirds the size of their normal litter mates. As a rule, the study of size inheritance is distinguished by the absence of clearly marked classes and there is no evidence of the clean-cut segregation that occurs in the inheritance of other traits. In the present instance, however, there is clear-cut segregation into

distinct classes and it is apparent that a unit factor is operative in the determination of size.

As a matter of fact, size inheritance of this type characterizes the history of the line in which the dwarfs occur. The Polish breed was originally derived from the Dutch and arose as a size mutation of a definite order. Moreover, first generation crosses between Polish and other larger breeds result not in a series of fairly uniform individuals of intermediate size, but in more or less definite classes of larger and smaller animals. A further distinct size class occasionally occurs in the breeding of non-dwarf transmitting Polish and is represented in the dwarf transmitting line by the semi-dwarf described above. It is conceivable, therefore, that the lethal dwarf represents the final stage in a series of size mutations leading to distinct classes of individuals whose size is of a measured order of reduction in relation to their forebears.

SUMMARY

An hereditary type of dwarfism in the rabbit has been described. In contrast to the dwarfs described in other animals, this type is evident at birth and conforms to the classification, nannosomia primordialis, as used in human pathology. In homozygous form the variation is lethal and produces a miniature individual approximately one-third the size of its normal sibs. Heterozygous animals are approximately two-thirds the size of normal sibs at birth and never attain an equal stature.

The expression of the variation is modified by genetic factors carried by a line of cretinoid animals and, rarely, dwarfs derived from crosses with this line survive for 1 to 2 months. The striking changes in such survivors are hypertrophy and hyperplasia of the acidophilic cells of the pituitary and atrophy of the gonads. Such changes are not present in ordinary dwarfs and it is concluded that the acidophilic hyperplasia represents the influence of the modifying factors of the cretinoid line and supplies the growth hormone responsible for survival. The gonadotropic hormone is not supplied by the secretory activity of these cells and as a result the gonads atrophy.

The evidence at hand indicates that the primary effect of the dwarfing gene is an inhibition of the secretory functions of the pituitary. In homozygous individuals, the inhibition is complete and the variation is expressed as a lethal dwarf. In heterozygous animals, the function of the organ is altered, producing an undersized individual. The modifying factors of the cretinoid line act either to partially remove the inhibition or to alter the constitution of the animal so that life is possible for a short period without the full complement of pituitary hormones.

BIBLIOGRAPHY

1. Greene, H. S. N., Hu, C. K., and Brown, W. H., *Science*, 1934, **79**, 487.
2. Castle, W. E., *J. Exp. Zool.*, 1929, **53**, 421.
3. Rosahn, P. D., and Greene, H. S. N., *J. Exp. Med.*, 1936, **63**, 901.
4. Hu, C. K., and Greene, H. S. N., *Science*, 1935, **81**, 24.
5. Greene, H. S. N., *J. Exp. Med.*, 1937, **65**, 809.
6. Greene, H. S. N., *J. Exp. Med.*, 1938, **67**, 369.
7. Greene, H. S. N., and Saxton, J. A., Jr., *J. Exp. Med.*, 1938, **67**, 691.
8. Rischbeth, H., and Barrington, A., Eugenics Laboratory Memoirs XV, parts VII and VIII, London, Dulau and Co., 1912.
9. Snell, G. D., *Proc. Nat. Acad. Sc.*, 1929, **15**, 733.
10. Sollas, I. B. J., *Rep. Evolution Com. Roy. Soc.*, 1909, **5**, 51.
11. Lambert, W. V., and Sciuchetti, A. M., *J. Hered.*, 1935, **26**, 91.
12. Smith, P. E., and MacDowell, E. C., *Anat. Record*, 1930, **46**, 249.
13. Zondek, B., *Lancet*, 1936, **1**, 10.

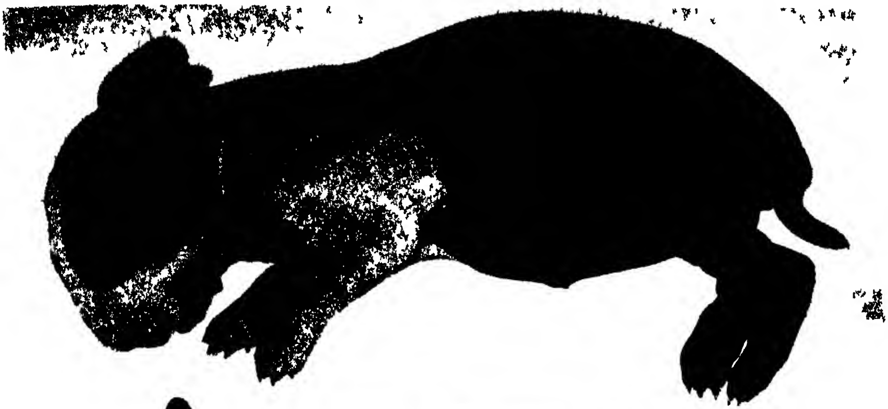
EXPLANATION OF PLATES

PLATE 48

FIG. 1. Photograph of litter mates taken at birth showing from top to bottom, a normal animal, a dwarf transmitter and a dwarf.

FIG. 2. The calvarium of a dwarf (lower figure) compared with that of a normal litter mate.

FIG. 3. Surviving dwarf and normal litter mate 20 days after birth.



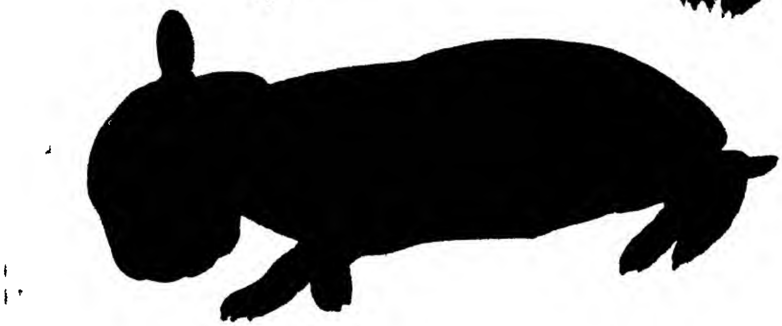
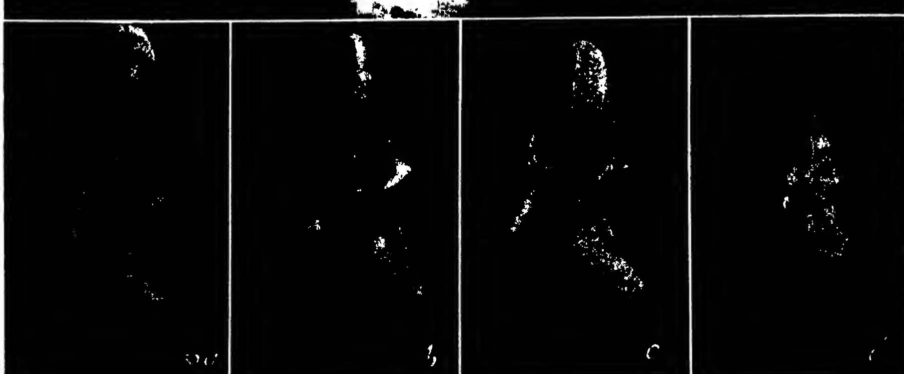


PLATE 49

FIG. 4. Surviving dwarf and normal litter mate 22 days after birth.

FIG. 5. Front view of animals pictured in Fig. 4.

FIG. 6. Calvaria of dwarf transmitters at different ages. *a*, adult. *b* and *c*, 5 months. *d*, birth. *a*, *c* and *d* show typical defects in the frontal bones. In *b* the defects have healed but irregular fissures persist in the region of the bregma.



Photographed by J. A. Carlile

Plate 50

Fig. 7. Section of the pituitary of a dwarf that survived for 14 days. The anterior lobe is almost completely replaced by hypertrophic acidophiles. Mallory's aniline blue. × 105.

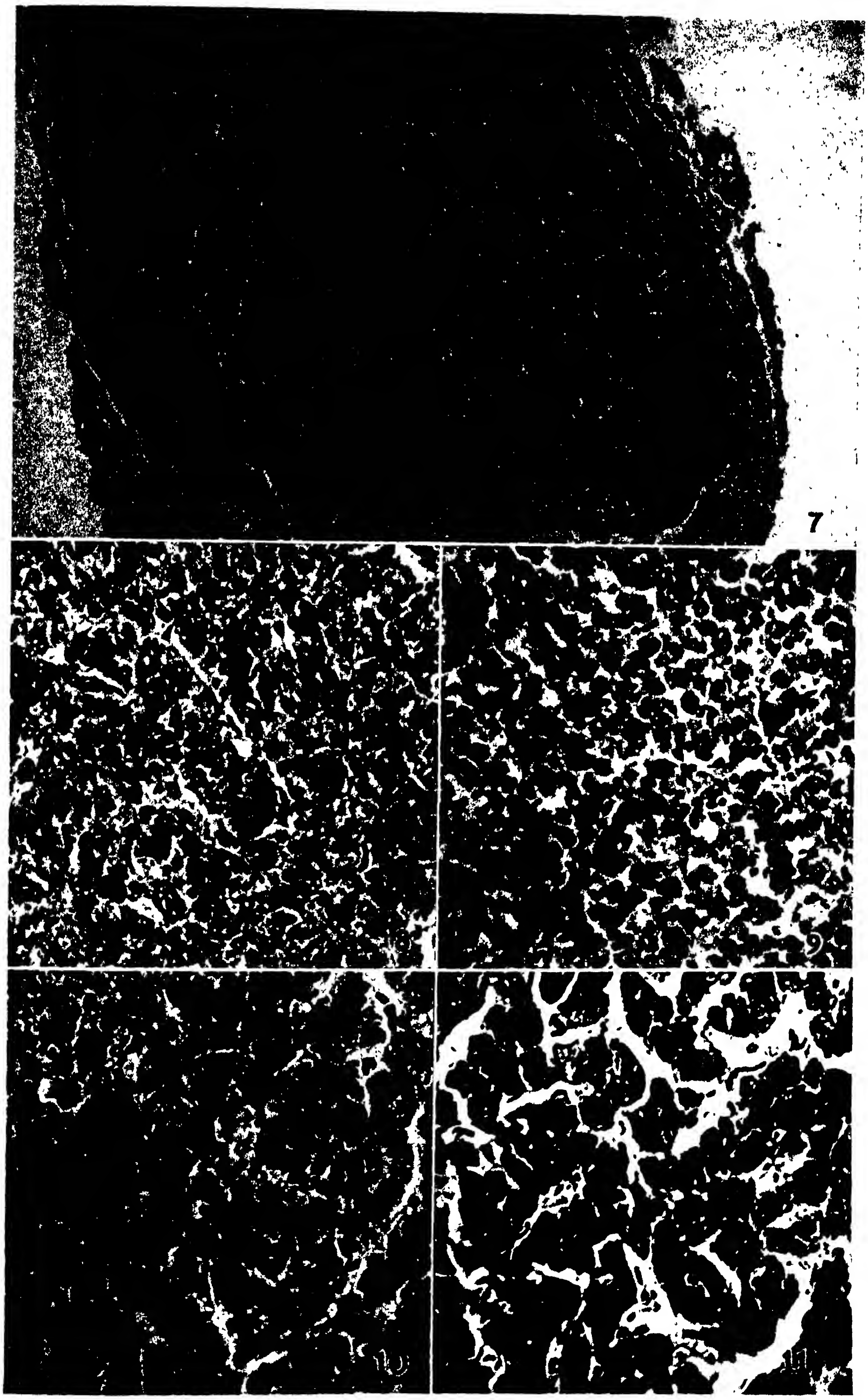
Fig. 8. Section of the anterior lobe of the pituitary of a dwarf that survived for 1 day. Acidophiles, basophiles and chromophobes are present in normal proportions. Mallory's aniline blue. × 196.

Fig. 9. Higher magnification of a section of the anterior lobe of the gland shown in Fig. 7. The lobe is made up almost entirely of hypertrophic acidophiles. Mallory's aniline blue. × 196.

Fig. 10. Section of the anterior lobe of the pituitary of the dwarf pictured in Fig. 3. This animal survived for 23 days. The great majority of cells are large acidophiles but rare basophiles and chromophobes are present. Mallory's aniline blue. × 196.

Fig. 11. Section of the anterior lobe of the pituitary of the dwarf pictured in Figs. 4 and 5. This animal survived for 26 days. The lobe resembled an acidophilic adenoma in the gross and high magnification revealed no other components than extremely large acidophiles. Mallory's aniline blue. × 196.

It should be noted that the gland shown in Fig. 8 was obtained from a dwarf whose ancestors did not carry factors concerned in the cretinoid abnormality, while those shown in Figs. 9, 10 and 11 were from dwarfs derived from crosses with the cretinoid line.



Photographed by J. A. Carlile

(Greene: Dwarf mutation in rabbit)

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ABSENCE OF VITELLINE MEMBRANES ON DEVELOPING EGGS IN PARASITIC FEMALES OF STRONGYLOIDES RATTI

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The alternative modes of larval development which occur in *Strongyloides ratti* Sandground, 1925, and apparently in all other species of the genus *Strongyloides* as well, have stimulated considerable investigation concerning the biological factors involved in producing this rather unusual phenomenon. In spite of the definitive points which have been established, resolution of the problem has been impeded, and clarification, even for *S. ratti*, is only at the penultimate stage.

Let us review the life cycle of *S. ratti* briefly. The parasite is normally found embedded in the mucosa of the small intestine in rats. Notably, only females have ever been observed. The eggs shed by the parasite complete their development in a fecal medium outside the body of the host. Some of the young larvae metamorphose directly into infective filariform larvae; others develop into morphologically distinct, sexually differentiated, free-living males and females which then produce a crop of infective filariform larvae morphologically indistinguishable from those that develop directly from the eggs of the parasite. These two modes of larval development are known as homogonic, or direct; and heterogonic, or indirect, respectively.

In its fundamental aspects the problem is essentially simple: How can one account for the production of two types of progeny by a *single* gynomorphic parasite, in one type of which sexual differentiation must also be accounted for? Confronted thus with the problem of determining whether parasitic *S. ratti* females reproduced parthenogenetically or syngonically, the writers, in addition to searching for hermaphroditically produced spermatozoa in the genital tubes of the parasite, have sought for evidence of fertilization in the eggs of this parasite.

Materials and Methods

Strongyloides ratti parasites from two pure lines with distinctive biological potentialities were used. In one instance the parasites were from stock

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infections which had been built up from a single, heterogonically derived parasite through the course of several generations by mass infections. The pure indirect line of single *S. ratti* from which these mass infections were derived was shown by Graham (1939) to produce predominantly progeny of heterogonic development, approximately 90 per cent of the total progeny being free-living males and females. On the other hand, the other line of *S. ratti* used in this study had been built up to a mass level from a pure direct line of singly established *S. ratti*. In this line (direct line of Graham, 1939, and direct line I of Graham, 1940a) only about 15 per cent of the total progeny were free-living males and females. Established at mass levels, these lines of *S. ratti* continued to show their respective characteristics; one yielded predominantly homogonic larvae, the other predominantly heterogonic adults.

Free-living females from the bisexual generation in the heterogonic line were also studied. These were used after they had developed in fecal cultures from 30 to 36 hours and 48 hours. Both fertilized and unfertilized eggs from free-living *S. ratti* females were also utilized in testing the reaction of reagents on shells and vitelline membranes.

A number of parasitic females of *Strongyloides* sp. from the common gray squirrel, *Sciurus carolinensis*, were also studied. Eggs from these parasites were likewise tested with reagents.

As comparative check material in the study of the developing eggs of *S. ratti*, eggs of the root knot nematode, *Heterodera marioni*, were employed. *H. marioni* eggs possess a readily observed vitelline membrane. Their reaction to reagents was reported by Chitwood (1938).

RESULTS

Observations were made on sealed saline mounts of parasitic *Strongyloides ratti* females with the specific objective of demonstrating spermatozoa in this reputedly syngonic form. In all instances parasites from 5 to 10 days old were studied. Being young, they were favorable material. Examinations were made immediately following their recovery from the intestine of the rat host. All were producing eggs in abundance and these were developing in an apparently normal manner. Using optical equipment which revealed details of structure with great clarity, prolonged search of the genital tubes of both intact worms and worms from which the internal organs had been extruded by cutting off the tail failed to reveal sperm cells.

Collateral observations made the absence of spermatozoa in these parasites assume a significant aspect. Polar bodies were observed in a number of eggs, but the majority of eggs examined did not show them. When they

were present, usually only one was seen in any egg. In several instances the polar body was at the distal end of the egg, but they were as frequently found at the proximal end of the egg or even in a lateral position. The usual pattern in nematode eggs which are fertilized is for more than one polar body to be thrown out, the first representing the meiotic division, being opposite the point of sperm entry, i.e., at the distal end of the egg. In the few instances in which two polar bodies were seen in an egg, they were always lying side by side, thus creating the impression that they had been formed by a division rather than representing the extrusion of two polar bodies from the egg. Walton (1918) has indicated that in *Toxocara canis* eggs rotation of the cytoplasm within the fertilization membrane may make it appear that the second polar body is given off at another position on the surface of the egg. While these observations in themselves are questionable evidence of non-fertilization, the random positions in which singly occurring polar bodies were seen support such a conception.

In one case the polar body was clearly resting against the egg shell. Ordinarily in fertilized nematode eggs, a fertilization or vitelline membrane is present and polar bodies are within, not outside, this membrane. Hence the observation of a polar body resting against the egg shell suggested the probable absence of such a membrane, unless it were closely adherent to the shell. Tests with various reagents failed to reveal a vitelline membrane. Eggs were rapidly stained with dilute iodine and with dilute gentian violet. In the presence of a vitelline membrane, penetration and staining of the egg mass would have been markedly delayed. Following treatment of the eggs with absolute alcohol (which is a solvent for the lipoidal vitelline membrane of nematode eggs), staining with iodine and gentian violet was not hastened. Heating also had no effect. This treatment melts and thus destroys the protective lipoidal vitelline membrane, permitting the entry of dyes. When "Clorox" (a 5 per cent solution of sodium hypochlorite which is a solvent for protein and chitin) was applied, the entire egg, including the shell, disappeared without leaving a trace of a vitelline membrane, which would have been undamaged by this reagent.

On the other hand, eggs of *Heterodera marioni* with a clearly visible vitelline membrane did not show staining of the embryo when treated with gentian violet. When treated with "Clorox" the shell was rapidly dissolved, but the embryo was protected by the vitelline membrane; however, when the "Clorox" was washed out with water and the shell-free eggs were treated with absolute alcohol, the vitelline membrane was quickly dissolved and the embryo freed. When eggs of *H. marioni* with shells were treated with absolute alcohol, then washed with water, and treated with dilute

gentian violet, the embryo was quickly stained by the dye, indicating that the membrane had been removed by the alcohol.

Although the presence of spermatozoa had been anticipated at the outset of these observations on parasitic *S. ratti* females (in part because of the rarity of parthenogenesis in nematodes and also because Sandground, 1926, had reported the presence of spermatozoa in parasitic *S. ratti* females), a thorough search failed to reveal them in this favorable material. The additional evidence that spermatozoa had not been present—namely, the observation of developing eggs in which a vitelline membrane could not be seen directly and no evidence of this membrane's presence found even when reagents were employed—indicated that these eggs were developing parthenogenetically. It is important to note that no distinction could be discerned either in parasites or in eggs from them irrespective of whether they were from a line producing a high percentage of free-living adults among their progeny or whether they were from the line producing predominantly direct development larvae.

On the other hand, examination of females from the free-living bisexual generation of *S. ratti* presented an entirely different picture. Feces were collected from rats harboring massive infections of *S. ratti* from a line producing predominantly adults of the heterogonic generation. Females isolated from fecal cultures incubated for 30 to 36 hours were studied. In many of them, numerous sperm cells were readily observed in the genital tubes at the junction of the uterus proper and the ovary. Polar bodies were at the distal end of developing eggs, not at the proximal end, i.e., the originally attached end, which is the characteristic, though not invariable, point of sperm entrance in bisexually reproducing nematodes. The vitelline membrane was easily seen even without the use of reagents. The membrane was destroyed by heating and treatment with absolute alcohol; eggs thus treated were rapidly stained by iodine and gentian violet, whereas the membrane in untreated eggs inhibited the entrance of these dyes. Treatment with "Clorox" dissolved the egg shell rapidly but left the vitelline membrane intact. This disappeared when it was heated or treated with absolute alcohol. Thus the tests with developing eggs from *S. ratti* females of the free-living bisexual generation were comparable to those with eggs of the root knot nematode, *H. marioni*, which also possess a vitelline membrane.

In those free-living females where sperm cells were not demonstrable (virgin or senescent individuals) no development of the eggs was seen and neither polar bodies nor vitelline membrane could be demonstrated by direct observation. Even when reagents were used for the latter the results

were negative. Thus the contrast between the condition in parasitic *S. ratti* females and *S. ratti* females from the free-living bisexual generation was remarkably distinct. Morphologically the detail of the genital tubes in these two types of *S. ratti* females is very similar. Reproductively they seem to pursue distinctly different lines. The free-living females follow the familiar nematode pattern, whereas the parasitic females, insofar as these observations can determine, are unusual in that their eggs possess no vitelline membrane and appear to develop parthenogenetically.

Studies of parasitic females of *Strongyloides* sp. from the gray squirrel, *Sciurus carolinensis*, were comparable in every respect to those made on *S. ratti*. No spermatozoa were seen in the genital tubes, although granules and cell clusters which might be mistaken for sperm cells were observed. Developing eggs did not possess a directly observable vitelline membrane nor could such membrane be demonstrated with the use of reagents. Polar bodies when present were usually single and were in random positions. In the few instances when two were observed in any egg they were always adjacent to each other. Apparently, parthenogenesis also occurs in the eggs of parasitic females of this form. Free-living females from the bisexual generation were not studied.

Unpublished observations by one of us (B. G. C.) indicate that the free-living nema, *Rhabditis filiformis*, for which only one sex is known, also reproduces by parthenogenesis. The developing eggs of this worm possess no vitelline membrane. To the writers' knowledge, these are the only known cases where evidence has been offered that parthenogenesis occurs in nematodes in the absence of spermatozoa, although it seems predictable that similar instances can be multiplied. The nearest approach to these cases is afforded by the free-living nematode, *Rhabditis aberrans*, in which parthenogenesis occurred even though sperm cells were present. Krüger (1913) observed insemination of ova in this protandrous hermaphrodite, but the male pronucleus did not fuse with the female pronucleus; instead it degenerated in the egg plasm without taking part in the formation of the embryonic nucleus. Under such conditions, only one polar body was produced. Apparently the only function of the spermatozoon—essential though it was for the development of the egg—was the initiation of cleavage. It should be noted that Krüger also observed *R. aberrans* to produce a very small percentage of male worms in cultures. Admitting the purity of the specific strain of the nematode with which she worked, the presence of a few males and the observation of a single egg in which two polar bodies and fusion of the male and female pronuclei were noted, suggest that parthenogenesis is not an exclusive feature in the reproduction of *R. aberrans*.

DISCUSSION

Certain historical aspects of the controversy concerning the reproductive status of parasitic females of various species of *Strongyloides* have been admirably presented and discussed by Sandground (1926). As he pointed out, Rovelli (1888) appears to have been the first investigator to examine parasitic *Strongyloides* females from the rat and subsequently report that they reproduced parthenogenetically. Unfortunately Rovelli's paper was inaccessible to Sandground and he depended on the accuracy and completeness of a review of it by Braun (1888). Granting this premise, Sandground's disposal of the reported evidence in favor of parthenogenesis was entirely justifiable. On the other hand, examination of the article by Rovelli leads to an impression different from that gained from Braun's review alone and forces one to keep in mind the possibility that parthenogenesis may occur in *Strongyloides* even if Rovelli's observations are not full proof thereof.

Rovelli (1888) stated that he made observations "sullo *Strongyloides* del *Mus decumanus* e su quello della Pecora"; not on "die Arten aus der Wanderratte und dem Schweine" as reported by Braun (1888). He noted further that there "resulterà chiaro dall'esame dell'apparato genitale (dalla sua origine fino alla estremità terminale, dalla iniziale formazione delle ova fino al loro completo sviluppo), non havvi giammai neppure un lontano sospetto della presenza degli elementi fecondatori maschili, e perciò mi credo autorizzato ad affermare (come incliniva ad ammettere il Grassi e contrariamente alle asserzioni del Leuckart) che *gli Strongyloides sono indubbiamente femmine partenogenetiche allo stadio di parassiti, mancando affatto il ricettacolo del seme e qualsiasi traccia di spermatozoi.*" (Italicized in the original.) Whatever deficiencies there may be in Rovelli's interpretation of his further observations, the statement above is clear and definite. While "this evidence is all of a negative character" as Sandground (1926) stated, it does not necessarily follow that "its reliability is not substantial." The writers believe that the absence of spermatozoa in parasitic *S. ratti* females studied by them is a "substantial" factual confirmation of Rovelli's observation, further fortified by the absence of vitelline membranes on the developing eggs.

It was in a definite attempt to account for the alternative modes of larval development on a "germinal chromosome basis" that Sandground (1926) "decided to re-investigate the sexual condition of the parasitic generation of Strongyloides." As he observed (p. 375), "In Strongyloides, the well established belief that the parasitic generation is represented by females

whose eggs develop parthenogenetically, has in the past automatically precluded the possibility of explaining the mechanism that determines the dual mode of development that this organism displays on the basis of an heterozygosis." Conceding the objectivity of his study in the course of which he examined "living specimens of *S. ratti*" and found spermatozoa "in appreciable numbers" at the point where the egg passes from the oviduct into the uterus, his observations stand in contrast to those in the present case in which no spermatozoa were encountered in any of the many worms examined. It is, of course, conceivable that he may have been dealing with a syngonic strain of the parasite differing from the parthenogenetic strains used in the present study. On the other hand, Sandground illustrated his report with photomicrographs (p. 348) of stained longitudinal sections of parasitic *S. ratti*. The caption of one of these states, "Shows oocytes and sperm cells; the tissue on the right of the section is packed with sperm cells, but at this focus they are only seen on the periphery." The section appears to have been cut somewhat obliquely and the cells at the periphery bear a striking resemblance to those of the uterine epithelium. Under such circumstances, it seems possible that the portion of the section which was "packed with sperm cells" may represent a frontal section of the cells of the uterine wall and not spermatozoa as he supposed.

Although we do not deny that Sandground (1926) may have seen spermatozoa in the living material of *S. ratti* which he examined, the present study of living *S. ratti* parasites failed to confirm the point. To the extent that his reproduced photomicrographs are fair objective evidence of what he found, his general conclusions in the matter must be considered with caution.

While the writers agree with Sandground (1926) that easy acceptance of the idea of parthenogenesis on the part of many previous authors was unfortunate and unjustified on a factual basis, it is equally unjustifiable to assume that it cannot occur. Thus Leuckart (1879) when confronted with "unverkennbare Samenkörperchen" in the uterus of *Ascaris nigrovenosa* [= *Rhabdias bufonis*], a monoecious parasite in the lungs of frogs, observed, "Ich muss übrigens hinzufügen, dass ich in manchen Fällen—und ebenso ist es auch andern Helminthologen ergangen (nach brieflicher Mittheilung z. B. v. Siebold)—vergebens nach Samenkörperchen gesucht habe, die Möglichkeit einer parthenogenetischen Entwicklung also noch keineswegs vollständig ausschliessen möchte."

The direct morphological evidence of parthenogenesis in parasitic *S. ratti* females herein reported is compatible with the observations made by one of us (G. L. G.) on pure lines of singly established *S. ratti*. Constitutionally

distinct lines of this parasite have been demonstrated (Graham, 1939; 1940a; 1940b). In those lines which were maintained by serial, homogonic passage (single larva infections), no evidence of constitutional deviation has ever been observed. On the other hand, serial passage in a heterogonically maintained line of singly established *S. ratti* has been shown to produce parasites on several occasions with biological and morphological characteristics which are constitutionally dissimilar from those of the parent from which they were derived. When these new lines with distinctive characteristics were maintained by serial homogonic passage, no discernible change was ever observed in succeeding generations of the parasite. These are facts which bear directly on the problem of determining the type of egg development in *S. ratti* of the parasitic generation. They provide no evidence that the parasite possesses a mechanism for inheritance of characteristics comparable to that observed in the free-living bisexual generation. Thus they obviously indicate the absence of males, at least functional males, in the parasitic generation and also deny the occurrence of hermaphroditic heterozygosis.

SUMMARY

Parasitic *Strongyloides ratti* females from a pure line producing predominantly progeny of homogonic development as well as from another pure line producing predominantly progeny of heterogonic development have been studied. In both cases, spermatozoa were not demonstrable in the genital tubes of these gynomorphic parasites nor could the presence of a vitelline membrane (evidence of fertilization) be shown in developing eggs of these parasites either by direct observation or the use of reagents.

On the other hand, sperm cells could be seen in free-living *S. ratti* females of the heterogonic generation and vitelline membranes seen in developing eggs as well as demonstrated by tests with reagents.

These results indicate that female *S. ratti* in the parasitic generation reproduce parthenogenetically whereas normal bisexual reproduction occurs in the free-living generation. Examination of parasitic females of *Strongyloides* sp. from the gray squirrel, *Sciurus carolinensis*, indicated that these parasites are reproductively comparable to parasitic *S. ratti* females, i.e., parthenogenetic.

BIBLIOGRAPHY

Braun, M. 1888 Review of article by Rovelli (1888). Centr. Bakt. 4: 660-661.

Chitwood, B. G. 1938 Further studies on nemic skeletoids and their significance in the chemical control of nemic pests. Proc. Helm. Soc. Washington 5: 68-75.

Graham, George L. 1939 Studies on Strongyloides. V. Constitutional differences between a homogonic and a heterogonic line of *S. ratti*. J. Parasitol. **25**: 365–375.

——— 1940a Studies on Strongyloides. VI. Comparison of two homogonic lines of singly established *S. ratti*. J. Parasitol. **26**: 207–218.

——— 1940b Studies on Strongyloides. VIII. Comparison of pure, related lines of the nematode, *Strongyloides ratti*, including lines in which gigantism occurred (in press).

Krüger, Eva. 1913 Fortpflanzung und Keimzellenbildung von Rhabditis aberrans, nov. sp. Z. Wissensch. Zool. **105**: 87–124.

Leuckart, R. 1879 Allgemeine Naturgeschichte der Parasiten. C. F. Winter, Leipzig u. Heidelberg. pp. X + 216.

Rovelli, G. 1888 Richerche sugli organi genitali degli Strongyloides (Anguillula, Rhabdonema) Fol. Como 12 pp.

Sandground, J. H. 1925 Speciation and specificity in the nematode genus Strongyloides. J. Parasitol. **12**: 59–80.

——— 1926 Biological studies on the life-cycle in the genus Strongyloides Grassi, 1879. Am. J. Hyg. **6**: 337–388.

Walton, A. C. 1918 The oögenesis and early embryology of *Ascaris canis* Werner. J. Morphol. **30**: 527–603.

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STUDIES ON STRONGYLOIDES

VI. COMPARISON OF TWO HOMOGONIC LINES OF SINGLY ESTABLISHED S. RATTI

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In *Strongyloides ratti*, a free-living bisexual generation may alternate with a parasitic generation composed only of females. Such a process has come to be called the heterogonic, or indirect, life cycle. On the other hand, a homogonic, or direct, life cycle may occur in which the progeny of the parasitic generation metamorphose directly into infective filariform larvae. The latter are then ready to infect a new rat host. These direct development larvae are morphologically indistinguishable from those produced by the free-living, bisexual generation.

There is a fundamental question involved in the life cycle of this parasite—and it applies to other species in the genus as well: Is the direction of development in the progeny of the parasite determined at the time the egg is formed or is it determined at a later time, i.e., is the destiny of the young larvae determined by the environment in which it develops? Beach (1936) contended that the mode of development of *S. simiae* larvae was not predetermined in the egg, but was governed by "influences" in their developmental environment. Faust (1936) considered it "significant" that this reported behavior on the part of *S. simiae* was "apparently not based on genetic factors," being contingent instead solely "on environmental factors."

With the demonstration by Graham (1935, 1936) that *S. ratti* could not only be established as a fertile parasite by exposing a rat to a single homogonic larva, but also maintained at this minimal level by serial passage, a new technical method for studying the bionomics of this parasite became available. On the basis of these studies it was established that in all phases the life cycle of *S. ratti* could be encompassed by the reproductive performance of a *single* parasite. Continued study of the daily reproductive activity of these singly established parasites produced evidence which made it difficult to believe that determination of the mode of larval develop-

ment was accomplished by influences acting on the larvae during their period of free-living development (Graham, 1938a).

When Graham (1939b) compared a homogonically derived line of singly established *Strongyloides ratti* with a similarly established, heterogonically derived line and pointed out biological characteristics which conclusively demonstrated the constitutional dissimilarity of these two lines, the idea of "environmental influence" as a critical element in determining the mode of larval development in this species became too heavily burdened with disharmonious facts to merit further serious consideration. While nothing is specifically known concerning possible "genetic factors," it is assumed that they may be involved in the observed "constitutional dissimilarity." Recognition of certain marked differences in the relative proportions of direct and indirect progeny between these two lines occurred with the establishment of the first *S. ratti* of heterogonic origin in a rat host, and continued serial passage brought other differences to light. This fact prompted an investigation of the potentialities of *homogonic* larvae derived from the initial parasitic generation of this *heterogonically* derived line.

In discussing the progeny relationships of a homogonically derived line of singly established *S. ratti*, Graham (1938a) stated that "evidence from the study of additional single larva infections of *S. ratti* of homogonic origin suggests that all homogonic larvae are not constitutionally equivalent." The factual basis for the above statement has since undergone considerable expansion, and data bearing on the point are herein presented in detail.

Materials and Methods

One homogonic line of singly established *S. ratti* has been studied for several years (Graham, 1935, 1936). In subsequent sections of this paper this line will be referred to as "direct line I." The new homogonic line, derived from the first parasitic generation of a heterogonically derived line and serially passed and studied in parallel with that line, will be referred to as "direct line II."

Descriptions of technical procedures with the homogonic line (direct line I) have been previously reported (Graham, 1936, 1938a and b, 1939a). Techniques used with the heterogonic line were presented in Graham, 1939b.

With the new homogonic line (direct line II) a combination of previous technical practices and precautions has been followed. The 24-hour fecal collections (daily cultures) were isolated after an incubation period of 24

hours, as were the cultures from the heterogonic line. This was necessary because of the heavy yields of free-living adults. Thus no confusion in enumerating and classifying the progeny as to mode of development occurred. With incubation periods of 48 and 72 hours reliably differentiated counts of the progeny were difficult to obtain due to the rapid development of filariform larval progeny of the free-living bisexual adults.

To obtain infective filariform larvae for the exposure of rats to a single homogonic larva each, cultures were isolated after an incubation period of 24 hours and the recovered progeny permitted to stand in tap water in 50 cc centrifuge tubes for an additional 24 or 48 hours. This procedure effectively inhibited the development of indirect larval progeny of the free-living adults while giving additional time to the larvae of direct development for their last ecdysis. This had seldom occurred after an incubation period of only 24 hours. As a further precaution against the use of non-infective larvae for the exposure of new rats, each larva used for infection purposes was examined carefully to ascertain that it had exsheathed.

The same strain of inbred laboratory rats was used as reported for previous studies in this series. The conditions under which those harboring single *S. ratti* of direct line II were cared for were the same as described by Graham (1939b) for the parallel heterogonic line as well as for that portion of the infections in direct line I with which calendar comparability was effected.

RESULTS

The characteristics of the singly established *S. ratti* in direct line II so closely resembled those of *S. ratti* in the parent heterogonic line from which this homogonic line was derived that it was impossible to distinguish between them on the basis of single infections. This was readily possible in the case of single *S. ratti* of the homogonic line, i.e., direct line I, and the heterogonic line (Graham, 1939b, Fig. 1). However, there was one point of difference which was of a magnitude and constancy permitting differentiation of the two series of infections, thus indicating that new characteristics may be revealed through the agency of heterogony in *S. ratti*.

In view of the several points of similarity between *S. ratti* of direct line II and the heterogonic line, marked dissimilarity of the two homogonic lines, i.e., direct lines I and II, was naturally evident. The points of difference between these two lines are the same as those enumerated by Graham (1939b) for *S. ratti* in direct line I and the heterogonic line, namely: (1) the relative yields of direct and indirect progeny, (2) the reproductive rate

(fecundity), (3) the length of reproductive life, (4) the frequency of production of free-living males of the bisexual generation, and (5) the infection rate.

Since repeated reference to the points of difference in the characteristics exhibited by *S. ratti* from a homogonic line (direct line I) and a heterogonic line (Graham, 1939b) has been and will be made, it seems desirable to provide a brief catalog of major items of difference. This is given in Table 1 which incorporates the data presented in Graham (1939b, Table 1) together with the additional data which accumulated for these two lines from July 1, 1938, until March 7, 1939, when study of the heterogonic line was discontinued. Changes between Table 1 (Graham, 1939b) and Table 1 of this study are thus quantitative in nature and serve to emphasize the fact that the biological differences between these two lines of *S. ratti* are constant.

TABLE 1

Comparison of Progeny Relationships and Other Points of Difference in a Homogonic Line (Direct Line I) and a Heterogonic Line of Strongyloides ratti from September 22, 1937, to March 7, 1939

	Direct line I	Heterogonic line
No. of parasites	34	25
Serial generations	20–57	1–12
No. of cultures with one or more offspring	2,559	1,229
Progeny		
Direct larvae	16,502	2,530
Indirect males	10	6,102
Indirect females	3,288	16,300
Total progeny	19,800	24,932
Percentage of indirect progeny	16.7%	89.9%
Ratio (approximate) of males to females	1:300	1:3

1. *Differences in the Relative Yields of Direct and Indirect Progeny*

The relative yields of the two progeny types, i.e., larvae of direct development and bisexual adults of the free-living generation, in the two lines under comparison (direct lines I and II) were markedly disproportionate. Evidence of this is shown in Table 2 in which it may be seen that only 16 per cent of the progeny secured from single *S. ratti* of direct line I were adults of indirect development, whereas 91 per cent of those obtained from the parasites of direct line II were indirect forms. It is to be noted that precise calendar comparability existed.

The average daily yield of each type of progeny calculated by weeks of parasitic age for 26 *S. ratti* in direct line II is shown in Fig. 1. In essential respects the curves almost exactly reduplicate similar curves for *S. ratti* of the parent heterogonic line (Graham, 1939b, Fig. 2). The impossibility of differentiating between these two lines of *S. ratti* on the basis of progeny type relationships alone is obvious. On the other hand comparison of the curves of Fig. 1 with similarly derived curves for *S. ratti* in direct line I (Graham, 1938a, Fig. 2, p. 228) shows the similarity of the curves of the principal progeny type but with the important distinction that in direct line I this type is of direct development whereas in direct line II it is of indirect development.

TABLE 2

Comparison of Progeny Relationships and Other Points of Difference in Two Homogonic Lines (Direct Lines I and II) of Strongyloides ratti from October 12, 1937, to September 10, 1938

	Direct line I	Direct line II
No. of parasites	25	26
Serial generations	31–47	1–13
No. of cultures with one or more offspring	1,666	1,798
Progeny		
Direct larvae	9,057	2,900
Indirect males	9	7,567
Indirect females	1,767	23,187
Total progeny	10,833	33,654
Percentage of indirect progeny	16.4%	91.4%
Ratio (approximate) of males to females	1:200	1:3

2. *Differences in the Reproductive Rate (Fecundity)*

It is evident from inspection of the curves mentioned in the preceding section that the reproductive rate of the *S. ratti* in direct line II and the parent heterogonic line must be very similar and that the rate of reproduction in these lines must differ somewhat from that of the *S. ratti* in direct line I. Graphic presentation, however, makes these points clearer. The cumulative progeny yields of Fig. 2 are derived (1) from data on direct line II as given in Fig. 1, (2) from data on direct line I (Graham, 1938a, Fig. 2), and (3) from information on the heterogonic line (Graham, 1939b, Fig. 2). Also included with the latter are data on the heterogonic line from July 1, 1938, to March 7, 1939. In each instance the weekly increment is based on the average progeny output from patent culture days to provide comparability.

The parallelism of the reproductive rate in the heterogonic line and in direct line II is clear as is the lower rate of reproduction which occurred in direct line I. At the end of 16 weeks (indicated in Fig. 2 by arrow) direct

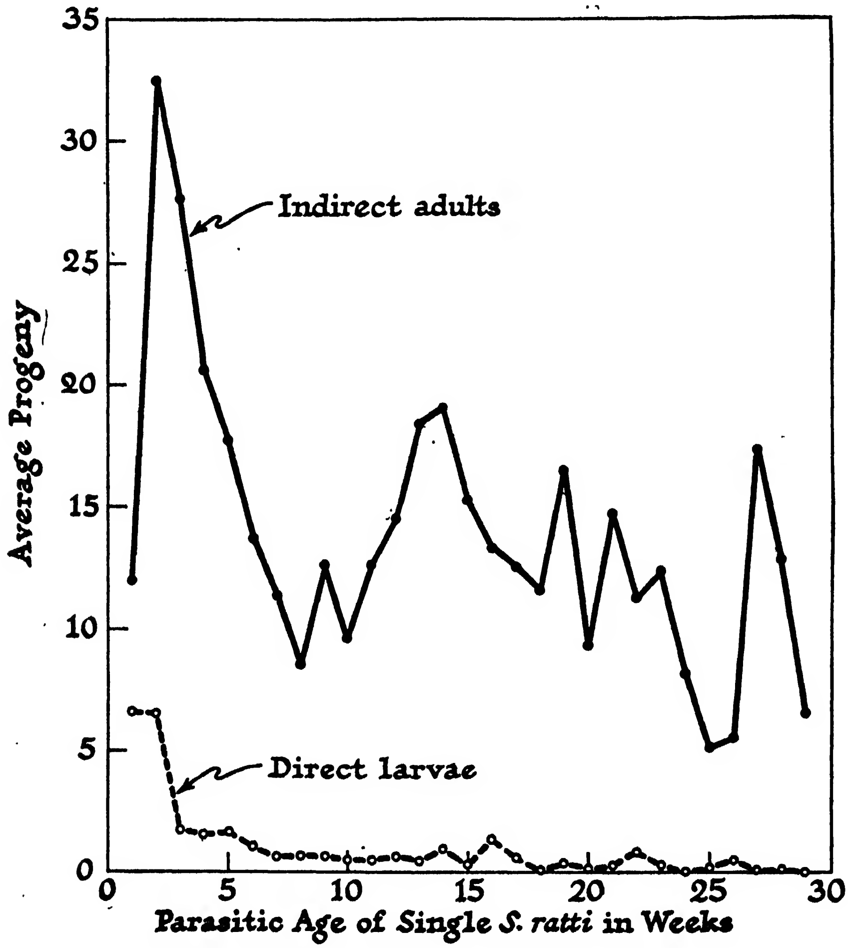

FIG. 1. Average number of free-living adults and larvae of direct development from 26 single *S. ratti* parasites in direct line II, as observed in cultures made from 24-hour fecal collections from the rat host.

line II reached a cumulative progeny level not attained by direct line I until 16 weeks later (indicated in Fig. 2 by arrow).

3. *Differences in the Length of Reproductive Life*

The length of reproductive life of singly established *S. ratti* of direct line I has been presented in detail (Graham, 1940). For 78 infections with good

end-points, i.e., 30 or more consecutive culture days without progeny after the last patent day of each infection, a mean reproductive life span of 149 ± 8 days was observed. In the heterogonic line, the length of reproductive life of *S. ratti* was distinctly less than this, being 11 weeks (Graham, 1939b). Actually, 17 infections with good end-points showed a mean life

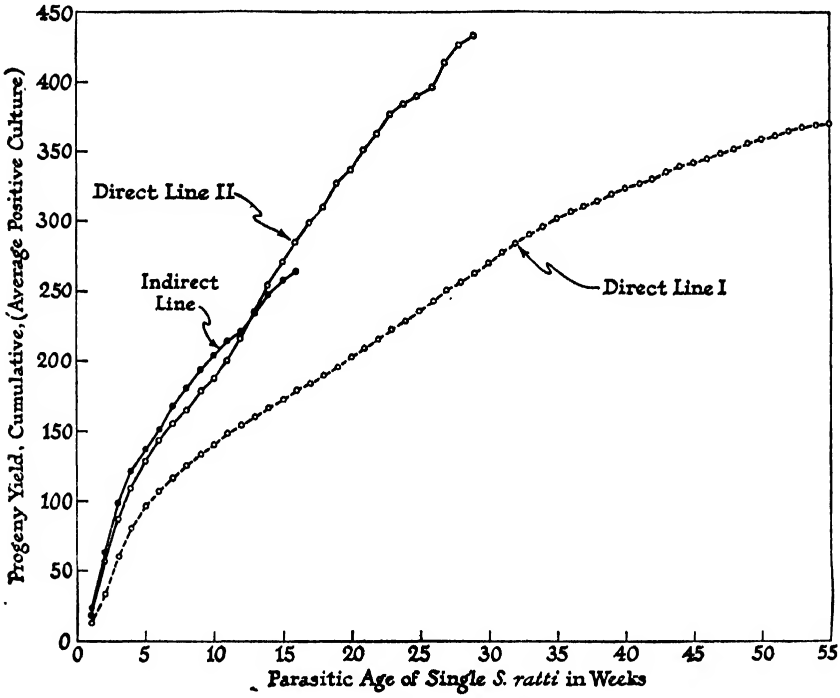

FIG. 2. The cumulative progeny yield from 26 *S. ratti* in direct line II, 116 *S. ratti* in direct line I, and 25 *S. ratti* in the heterogonic line (from which direct line II was derived). Weekly accumulations are comparable, being based on the average yields of positive cultures. In direct line II, 1,798 culture days yielded 33,654 progeny; in direct line I, 5,118 culture days yielded 54,867 progeny; in the heterogonic line, 1,229 culture days yielded 24,932 progeny.

span of 72 ± 4 days. The difference in the length of reproductive life in these two lines is significant statistically.

In direct line II, as may be observed in Fig. 2, the *S. ratti* were reproductively longer lived than those in the heterogonic line but shorter lived than those in direct line I. Twenty-two infections for which good end-points of reproductive activity were obtained showed a mean life span of 90 ± 8 days. The differences in the mean reproductive life spans of *S. ratti* in direct lines I and II are statistically significant, but the differences

between the heterogonic line and direct line II are insignificant. It is possible of course that this may be accounted for by the small number of infections in these two lines. Regardless of mathematical considerations, the fact remains that several of the *S. ratti* in direct line II were reproductively active for a considerably longer period (the longest life span in the group being 199 days) than any in the parent heterogonic line. Prolonged observation of additional parasites in these lines might show valid statistical differences in the length of their reproductive lives.

4. *Differences in the Frequency of Production of Males of the Free-Living Bisexual Generation*

As in the heterogonic line, the rate of production of free-living males in direct line II was high, being 22.5 per cent of the total progeny obtained from this line. In the heterogonic line 24.5 per cent of the total progeny were free-living males. On the other hand, the frequency with which free-living males were observed in direct line I was almost negligible, being less than 0.1 per cent of the total progeny observed.

Attention is directed to the similarity of the ratio of approximately 3 females to 1 male in the bisexual progeny from *S. ratti* in the heterogonic line and direct line II, as shown in Tables 1 and 2. (Actual ratios were 2.7 in the former and 3.1 in the latter.)

5. *Differences in Rate of Infection*

An infection rate of 26.2 per cent was reported from the exposure of 668 rats to a single homogonic larva each in direct line I (Graham, 1939b). During more than 4 years since the first infections in this line were initiated, the cumulative infection rate has varied but slightly. A similar constancy has likewise been noted in the cumulative infection rates in the heterogonic line and direct line II ever since these two lines were established. In the heterogonic line, which was passed through 12 serial generations, 64 rats were exposed to a single larva each and 25 infections (39 per cent) obtained. In direct line II, derived from the heterogonic line, the exposure of 35 rats to a single homogonic larva each during passage of the line through 13 serial generations yielded 26 (74 per cent) positive infections. The writer's confidence in the high infection rate in this line became so great that in a number of instances only a single rat was exposed to a larva for establishing a new generation, and only once was failure encountered in doing so.

As pointed out earlier (Graham, 1939b), the difference in the infection rates in the heterogonic line and direct line I (39 and 26 per cent, respectively) was not sufficiently great and the number of exposures in the hetero-

gonic line was too few to permit of statistical validity even if the difference was actually real. However, another situation prevails in the case of the high infection rate obtained in direct line II even though the number of exposures was small. Here it can definitely be stated that there is only one chance in 200 that the true infection rate might be lower than 50 per cent or higher than 90 per cent. In the heterogonic line there is only one chance in 200 that the true infection rate might be lower than 25 per cent or higher than 55 per cent. While there is a slight overlapping of these limits, the statistical odds definitely favor the interpretation that the dissimilar infection rate in the *S. ratti* of these two closely related lines is real rather than apparent.

Although these two lines of singly established *S. ratti* may be so similar in other characteristics (with the possible exception of length of reproductive life) that they are indistinguishable, on this one point, namely, in rate of infection, they appear to be consistently, as well as statistically, dissimilar. The dissimilarity seems accountable only on the basis of constitution.

The difference in the infection rates in direct lines I and II is statistically valid and serves to re-emphasize the now well established fact that "all homogonic larvae are not constitutionally equivalent," as noted by Graham (1938a).

DISCUSSION

The points of difference between the two homogonic lines of singly established *S. ratti* reported herein were clear cut and demonstrated that homogonic larvae of *S. ratti* may differ constitutionally. Far more significant is the fact that these lines, once established, are maintained without apparent change by continued homogonic passage. The seasonal variation in the frequency with which culture days yielded adults of indirect development in direct line I (Graham, 1939a) is not comparable in degree to the progeny type characteristics herein accepted as criteria for differentiating these distinctive lines of *S. ratti*. Constancy of distinguishing characteristics was likewise observed in another homogonic line of *S. ratti*, which was derived from the same heterogonic line of *S. ratti* as direct line II. The details of this additional line will be reported in another paper.

Although the evidence is limited in that only a few lines have so far been demonstrated and studied, the fact that any distinctive lines of homogonically derived *S. ratti* can be recognized makes it seem odd that these lines arise only by way of heterogony and never from a parasitic female by way of homogony. Their constitutional dissimilarity seems clearly established. If, as Sandground (1926) claimed, *Strongyloides ratti* actually

is a syngonic organism, and possesses, as it would appear, a variety of segregable characteristics, one may ask the question: why do these pure homogonic lines exhibit such constancy in their characteristics and why do not new lines exhibiting distinctive characteristics arise from them? Inferentially, one possible explanation is that "homogony" is essentially a vegetative mode of propagation achieved without fertilization of the ova. The observation of Chitwood and Graham (1940) to the effect that parasitic *S. ratti* are parthenogenetic forms seems significant in this connection.

The contrast between the results of the present study and the mass selection experiments of Sandground (1926) with *S. ratti* is extreme. In passage through 10 serial indirect generations Sandground observed but one instance in which free-living adults exceeded larvae of direct development among the progeny. In 12 mass infections in a direct development series of 11 serial generations none produced more indirect than direct progeny, thus being somewhat comparable to direct line I of this study. Sandground observed: "If the original infection, displaying both modes of reproduction with a preponderance of direct development, be regarded as a type characteristic, it is seen that this characteristic is conserved or transmitted through the medium of larvae of direct development." Insofar as his experiments with *S. ratti* are concerned this principle seems to apply almost equally well to larvae of indirect development.

With *Strongyloides papillosus* in the rabbit, Sandground (1926) observed preponderantly direct development in infections established with larvae of direct development obtained from a sheep infection. When selection for indirect development from these homogonically established infections was made, infections were obtained which showed preponderantly indirect development. After passage of this heterogonic line through several generations, an infection established in a rabbit with homogonic larvae derived from a parental generation established heterogonically showed predominantly indirect development. Concerning this, Sandground observed that the result was "rather unexpected" although "consistent with the principal enunciated earlier" on the basis of his *S. ratti* selection experiments. Thus with regard to mode of larval development the relationship existing between direct line II and the parent heterogonic line bears a resemblance, at least superficially to that observed by Sandground in the case of *S. papillosus* in rabbits. Closer comparison is unwarranted.

It would be interesting to know the extent to which homogonic larvae from a heterogonically established mass infection which could develop into parasites capable of producing predominantly progeny of direct develop-

ment might be masked by more highly infective larvae with different potentialities concerning the mode of larval development. It seems possible that some generalizations concerning *Strongyloides* are not safely based on mass infection experiments.

Comparatively little is known concerning the extent to which varieties or strains of nematode species occur in nature. What meager evidence is available bears in part on strain differences as measured in two host species, rather than in a single one. Some plant parasitic nemas may become so well adapted to new host plants that it is with difficulty that they can become re-adapted to a previously favorable host species. Among nematodes parasitic in vertebrates only a few instances of variation in physiological characteristics have been reported. Scott (1929, 1930) demonstrated the existence of two strains of the hookworm, *Ancylostoma caninum*, which showed a high degree of specificity for the dog and cat respectively. On the basis of egg-worm ratios Graham and Porter (1934) showed that a strain of *Nippostrongylus muris* from Texas consistently produced fewer eggs per female than two strains of the parasite recovered from wild rats at Baltimore.

Chandler (1932) suggested "that a parasite may become better adapted to a host other than the normal one," believing that in the course of repeated passage through white laboratory rats *N. muris* became better adapted to the latter than to their normal wild rat hosts. In the experience of Graham and Porter (unpublished data) laboratory rats were found to be at least as favorable hosts for *N. muris* as wild rats. Taylor (1928) found that when the gapeworm *Syngamus trachea*, derived from starlings, was passed through one generation in chickens, the infectivity of the parasite for the latter host was increased, although later Clapham (1935) readily infected chickens with gapeworms of starling origin. Thus the evidence for adaptation among animal parasites is slight, although it seems probable that any change in host strains or host species should lead automatically to selection of characteristics in the parasite both favorable and unfavorable for survival. It seems unlikely that such changes in the constitution of parasites in mass infections would be detected readily unless the changes were of considerable magnitude.

In the case of *Strongyloides ratti* the differences which have been noted were obtained under extremely favorable conditions in a single host species which was a highly inbred and therefore a relatively homogeneous line. These differences were thus directly measurable and referable to the constitution of the parasite.

SUMMARY

1. A homogonic line of singly established *S. ratti* (direct line II) showed remarkable similarity in most respects to the heterogonic line from which it was derived but was distinguishable from the parent line on the basis of a higher infection rate. Direct line II was compared with another homogonically maintained line of singly established *S. ratti* (direct line I) from which it differed in a number of respects.

2. Direct line II differed from direct line I in producing predominantly progeny of indirect development (91 per cent), in showing a higher reproductive rate, in being shorter lived, in producing many more males of the free-living generation, and in producing filariform larvae which were nearly three times as infective when exposed singly on rats.

3. The characteristic differences between these two homogonic lines of singly established *S. ratti* indicate clearly that all homogonic larvae of *S. ratti* are not constitutionally equivalent.

4. The characteristics of these homogonically maintained lines of *S. ratti* appear not to be changed during the course of serial homogonic passage.

5. On the basis of present evidence it appears that new lines of *S. ratti* arise only through the agency of heterogony and never by homogony.

BIBLIOGRAPHY

Beach, T. D. 1936 Experimental studies on human and primate species of Strongyloides. V. The free-living phase of the life cycle. Am. J. Hyg. **23**: 243-277.

Chandler, A. C. 1932 Experiments on resistance of rats to superinfection with the nematode, *Nippostrongylus muris*. Am. J. Hyg. **16**: 750-782.

Chitwood, B. G., and Graham, G. L. 1940 Absence of vitelline membranes on developing eggs in parasitic females of *Strongyloides ratti*. J. Parasitol. **26**: 183-190.

Clapham, P. A. 1935 On the experimental transmission of *Syngamus trachea* from starlings to chickens. J. Helm. **13**: 1-2.

Faust, E. C. 1936 Strongyloides and strongyloidiasis. Rev. Parasitol., Clin. y Lab., Habana **2**: 315-341.

Graham, G. L. 1935 Single larva infections of *Strongyloides ratti* Sandground, 1925, as an approach to certain problems of Strongyloides bionomics. (Abstract) J. Parasitol. **21**: 432.

——— 1936 Studies on Strongyloides. I. *S. ratti* in parasitic series, each generation in the rat established with a single homogonic larva. Am. J. Hyg. **24**: 71-87.

——— 1938a Studies on Strongyloides. II. Homogonic and heterogonic progeny of the single, homogonically derived *S. ratti* parasite. Am. J. Hyg. **27**: 221-234.

——— 1938b Studies on Strongyloides. III. The fecundity of single *S. ratti* of homogonic origin. J. Parasitol. **24**: 233-243.

——— 1939a Studies on Strongyloides. IV. Seasonal variation in the production of heterogonic progeny by singly established *S. ratti* from a homogonically derived line. Am. J. Hyg. **30** Sec. D.: 15-27.

Graham, G. L. 1939b Studies on Strongyloides. V. Constitutional differences between a homogonic and a heterogonic line of *S. ratti*. J. Parasitol. **25**: 365–375.

——— 1940 Studies on Strongyloides. VII. Length of reproductive life in a homogonic line of singly established *S. ratti*. Rev. Med. Trop. y Parasitol., Bact., Clin. y Lab., Habana (in press).

Graham, G. L., and Porter, D. A. 1934 Strains of *Nippostrongylus muris* and their behavior in various strains of rats. (Abstract) J. Parasitol. **20**: 323.

Sandground, J. H. 1926 Biological studies on the life-cycle in the genus Strongyloides Grassi, 1879. Am. J. Hyg. **6**: 337–388.

Scott, J. A. 1929 Experimental demonstration of a strain of the dog hookworm, *Ancylostoma caninum*, especially adapted to the cat. J. Parasitol. **15**: 209–215.

——— 1930 Further experiments with physiological strains of the dog hookworm, *Ancylostoma caninum*. Am. J. Hyg. **11**: 149–158.

Taylor, E. L. 1928 *Syngamus trachea* from the starling transferred to the chicken, and some physiological variation observed. Ann. Trop. Med. and Parasitol. **22**: 307–318.

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STUDIES ON STRONGYLOIDES

VII. Length of Reproductive Life in a Homogonic Line of Singly Established S. ratti

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In the course of other studies on *Strongyloides ratti* (Graham, 1936, 1938a, 1938b, 1939a, 1939b) data relating to the length of reproductive life of *S. ratti* in an essentially pure line have been obtained. These longevity determinations were made on parasites established singly in laboratory rats. This simplification of the infection level has afforded an opportunity for the study of parasitic length of life of this nematode uncomplicated by factors such as acquired resistance, which is known to shorten the period of parasitic life in mass infections.

The length of reproductive life and the whole life span of these single *S. ratti* may be identical, although no evidence is adduced for or against such a view in the present study. The post-patent period of examination was sufficiently extended in most instances to establish accurately the end of the reproductive period. It is assumed that the cessation of reproduction is a practical measure of the length of life of these singly established parasites. No attempt was made to recover any of the post-patent single *S. ratti* by direct examination at autopsy.

Materials and Methods

Each of the 161 *S. ratti* infections which have been observed was established by a single larva of direct development. All but 26 infections (Graham, 1939a) were from a pure line which was maintained by serial passage.

Five of the 161 infections were not included in this analysis because they were "patent" only on a single day (respectively days 6, 7, 11, 12, and 13 after exposure of the rat host to a single infective larva). The recovery of a single direct development larva on the indicated day was the only evidence of infection. In these five instances it was impossible to deter-

mine whether the single larva recovered from the fecal culture was the one employed to infect the rat host or whether it was a single offspring of the respective single parasites which were lost almost as soon as they reached maturity. The conservative view is taken that in these five instances the "infecting" larvae were themselves being observed. The arbitrary discarding of these five questionable infections is of little consequence insofar as conclusions are concerned.

Five other infections have been observed which were patent on only a single day (respectively days 6, 6, 8, 9, and 10 after infection), but more than one offspring was observed from each infection, indicating with certainty that the single parasite had matured. On another occasion, an infection produced a single free-living female on day 6 after infection and a single direct development larva on day 19.

About one-half of the single *S. ratti* infections were followed by consecutive daily 24-hour fecal collections from the rat host (Graham, 1936) during the late patent and post-patent period to determine accurately the end-point of reproductive activity. Failure to demonstrate larvae in such cultures for 30 or more consecutive days was accepted as evidence that the single parasite, if still in the intestine of the rat, was reproductively exhausted.

The arbitrary selection of 30 days as an acceptable period of negative examination for determining the end of reproductive activity was based on two considerations: (1) the necessity of setting a practicable limit which would permit observations on a larger series of single *S. ratti* than would otherwise have been possible, and (2) familiarity with the premonitory signs in the reproductive performance of these single *S. ratti* which were noted (Graham, 1938b, p. 238) as indicating the approaching end of patency. Actually the period of negative examination was raised progressively from 14 days to 20 days, then to 25 days, and finally to 30 days in an effort to establish the end of patency at an accurate and practicable level.

Reference to the length of "reproductive life" or "life span" of these singly established *S. ratti* is to be understood as dating from the day of infection until the last 24-hour fecal collection was obtained which yielded progeny. The prepatent period as well as the known patent period is thus specifically included.

RESULTS

Of the 156 infections which are admitted for consideration, only five are now patent. For analytical purposes the balance has been divided into two groups: 78 infections for which good end-points, i. e., 30 or more con-

secutive negative days, were established; and 73 infections with no end-points, poor end-points or questionable end-points. The latter group included 37 *S. ratti*, the rat host of which either died of natural causes or was discarded while the infections were still patent.

The most important group and also the largest is that for which well established end-points are available. The length of reproductive life of these 78 singly established *S. ratti* ranged from 8 to 477 days with a mean reproductive life span of 149 ± 8 days. In the group of 73 *S. ratti* with inadequate end-points the range in the length of reproductive life was from 5 to 484 days and the known average life span was 89 days.

Distributionally these two groups present a similar picture with regard to known length of reproductive life. A distribution diagram showing the known life span of the *S. ratti* in each of these groups is presented in Fig. 1. The dotted line diagram of the unselected group with inadequate end-points clearly shows the effect of including infections which were discarded early in the patent period. Intermittent examination which resulted in poor end-points accounted for many of the infections of medium duration. The longer-lived infections in this group were almost entirely cases in which the rat host died while the *S. ratti* were still patent or the sequence of negative examinations was short of the accepted number.

While analysis of the length of reproductive life of single *S. ratti* must hinge primarily on the larger group with good end-points, considerable supplemental value attaches to the group with poorer end-points.

Simplification of the data for analysis is effected by grouping. To the 78 infections in the group with good end-points have been added five from the group with inadequate end-points, but in these five instances the end-points were definite within specific limits. These five infections were reproductively active for 88 (9), 90 (3), 96 (9), 166 (4), and 273 (10) days respectively. The figures in parentheses indicate the number of days which elapsed from the last positive culture until fecal collections were resumed which resulted in a series of negative cultures. All of these negative series were of accepted length, i. e., 30 or more days. With the exception of the last, all fall within the specific parasitic age groups used in Table 1 even when cognizance is taken of the indeterminant period.

In Table 1 these 83 infections are distributed by 5 week intervals indicating the length of reproductive activity. It will be seen that the percentage of infections which became reproductively inactive increased rapidly during the first 20 weeks of parasitism, then declined rather sharply and flattened out at a low level. As shown in the last column of Table 1, 44

per cent of the infections were still patent at the end of 20 weeks of parasitism. At the end of 40 weeks only 11 per cent were reproductively active.

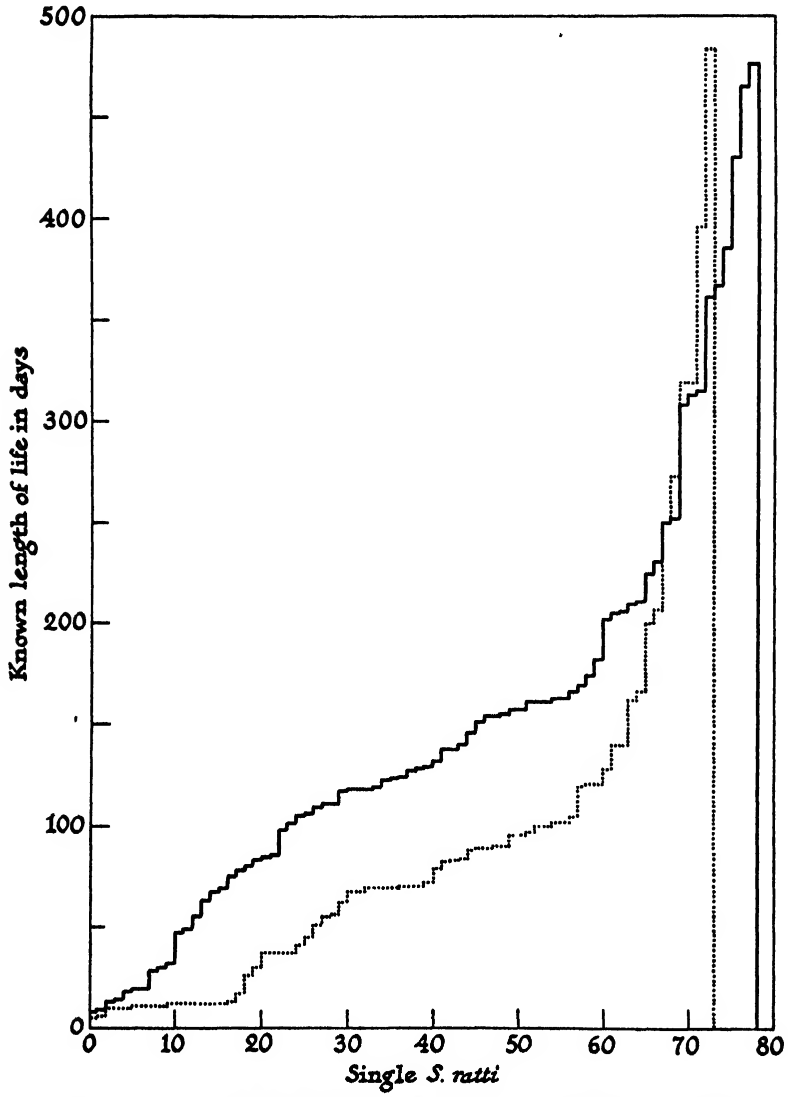

FIG. 1. Distribution diagram showing the length of reproductive life of 78 single *S. ratti* with well determined end-points (solid line) and 73 single *S. ratti* with inadequately determined end-points (dotted line).

The frequency with which these single *S. ratti* infections reached a negative status is shown in Fig. 2, which reproduces graphically the data from columns 2 and 3 of Table 1. As indicated in the graph the median length

of reproductive life of these 79 infections was 18 weeks. The median (18 weeks) and mean (149 ± 8 days) values closely approach the modal value (15–20 weeks). The distribution of these frequencies is such that the curve described by them roughly approximates a normal distribution, although the great length of life of a few *S. ratti* leads to some skewness. It would appear that the length of life of the single *S. ratti* in this group was to a considerable extent determined by factors operating at random and not necessarily related to reproductive capacity. Thus, the fecundity of

TABLE 1

Showing the Number and Percentage of 83 Single S. ratti Which Became Post-Patent in Consecutive 5 Week Periods and the Percentage of the Original Infections Which Remained Patent at the End of Each Period

(See Fig. 2)

Period of parasitic age	Single *S. ratti* becoming post-patent during period		Single *S. ratti* remaining patent
Weeks	*Number*	*Per cent*	*Per cent*
1– 5	10	12	88
6–10	6	7	81
11–15	12	14	67
16–20	19	23	44
21–25	16	19	25
26–30	5	6	19
31–35	3	4	15
36–40	3	4	11
41–45	3	4	7
46–50	0	0	7
51–55	2	3	4
56–60	1	1	3
61–65	1	1	2
66–70	2	2	0
Total..............	83	100	—

shorter-lived *S. ratti* in this line was not perceptibly different from longer-lived *S. ratti* at comparable ages.

The data in column 4 of Table 1 express as a percentage balance, the remaining patent infections of the 83 single *S. ratti* infections at the end of consecutive 5 week intervals. When plotted on arithlog paper, these data produced a curved line instead of the straight line which occurs with data fitting an exponential curve. The latter signifies a constant rate of loss, which appears to characterize the decrease in the worm burden in mass infections of human hookworm (Chandler, 1926), *Ancylostoma braziliense*

in the cat (Sarles, 1929a), *A. caninum* in the dog (Sarles, 1929b), *Trichostrongylus calcaratus* in the rabbit (Sarles, 1932), and *Heterakis spumosa* in the rat (Winfield, 1933). Loss of worms from these mass infections are indicated as differing biologically from the result with single *S. ratti*.

One point is clearly discernible in Fig. 2, namely, that a few of these single *S. ratti* remain reproductively active over a long period. The three oldest infections that have been observed remained fertile for 466, 477, and 484 days respectively. Four others were patent for more than a year. It is

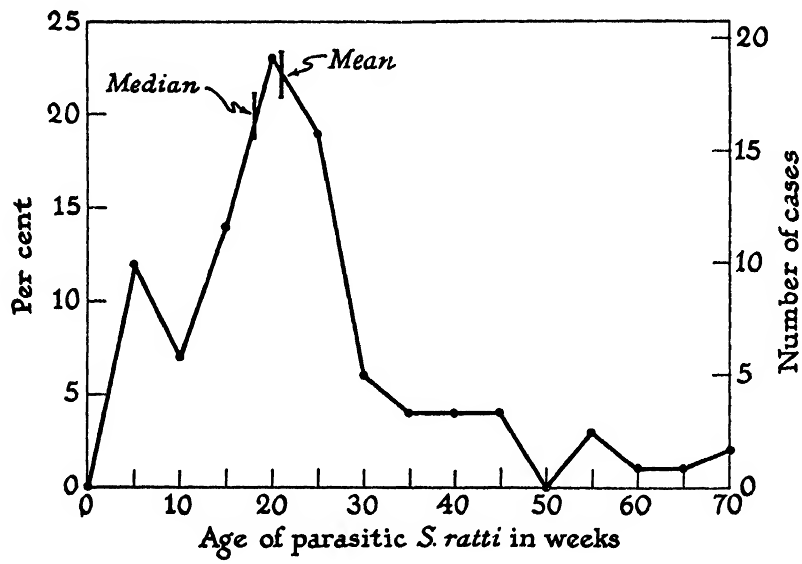

FIG. 2. Frequency distribution curve indicating the percentage of 83 single *S. ratti* which became reproductively inactive during consecutive five week periods. Cessation of reproductive activity based on 30 or more consecutive days of negative examination.

important to note that no evidence of hyperinfection has ever been observed in any of these single *S. ratti* infections. Consequently this hypothetical phenomenon cannot be invoked as a possible explanation for the extended longevity of these infections.

Basing the length of life of singly established *S. ratti* on the cessation of reproductive activity leads necessarily to an examination of evidence indicating that reproduction in these single parasites has actually ended. Among the possibilities which must be considered are: (1) that the single worm has died or been lost from the intestine while eggs deposited in galleries in the mucosa or circulating in the cecal "whirlpool" continue to be shed for some time; (2) that reproduction has only ceased temporarily; and

(3) that intermittent periods of negativity are indicative of internal or hyperinfection and the evidence herein assembled entirely inadmissible concerning the length of life of single *S. ratti*.

The latter point can be dismissed briefly. Rats harboring single *S. ratti* which had been reproductively inactive for 30 or more days were re-infected with a single parasite each. Patency ensued within an appropriate time. The resulting progeny yields were heavy and characteristically like those obtained from young *S. ratti* in initial infections. On the other hand the progeny yields obtained from single *S. ratti* after a period of negative culture days never resembled those obtained from these re-infections with single *S. ratti*.

It is impossible to know the amount of lag which may occur between final oviposition by the parasite and the final shedding of eggs or larvae from the intestine of the rat host. Scott (1928) showed that infective larvae of *Ancylostoma caninum* introduced into rats by means of a stomach tube remained in the intestinal tract apparently unchanged for 21 days. In the group of 78 single *S. ratti* infections with good end-points, five infections were negative for periods varying from 21 to 26 days before positive cultures were procured which prolonged patency. If these infections had been discarded with negative examination periods of 21 days their known patent period would have been shortened by 77, 78, 81, 88, and 216 days respectively. It does not seem likely that prolongation of the patent period can be accounted for in these cases on the basis of intestinal lag. Neither can it be attributed to hyperinfection or re-infection, for upon resumption of patency the low progeny yield was typically that of an old parasite in each instance. Insofar as can be determined it appears that *S. ratti* may be reproductively quiescent for periods of 3 weeks or more. It is possible that an occasional infection might show a long negative period which was due to intestinal lag in elimination of the last progeny from a lost or dead *S. ratti*. On the basis of present evidence, however, a long lag period must be judged as unusual.

In following the course of the 78 infections for which acceptable end-points were eventually secured, 1148 negative sequences of from 1 to 26 days' duration were observed. Negative periods of from 1 to 10 days' duration accounted for 98 per cent of this total and periods of from 1 to 4 days, 90 per cent. This clearly shows the infrequency with which long negative sequences occurred during the patent period.

On the basis of these data it has been possible to establish a remarkably smooth curve expressing as a percentage the probability that a negative sequence of a given number of days duration will continue negative for a

minimum of 30 days. This is shown in Fig. 3. With a negative sequence of 7 days' duration the chances are about even that an infection will continue negative for 30 days rather than become positive again. With a negative sequence of 11 days' duration, chances are 4 to 1 that the infection will continue negative to 30 days. At 14 days the chances are 9 to 1 and at 21 days 19 to 1 that a 30 days negative sequence will result. The steady rise of this curve to the 13 day level, with the subsequent flattening, reflects the adequacy of a 30 day consecutive sequence for determining the end of patency in single *S. ratti*. However, a 30 day negative examination

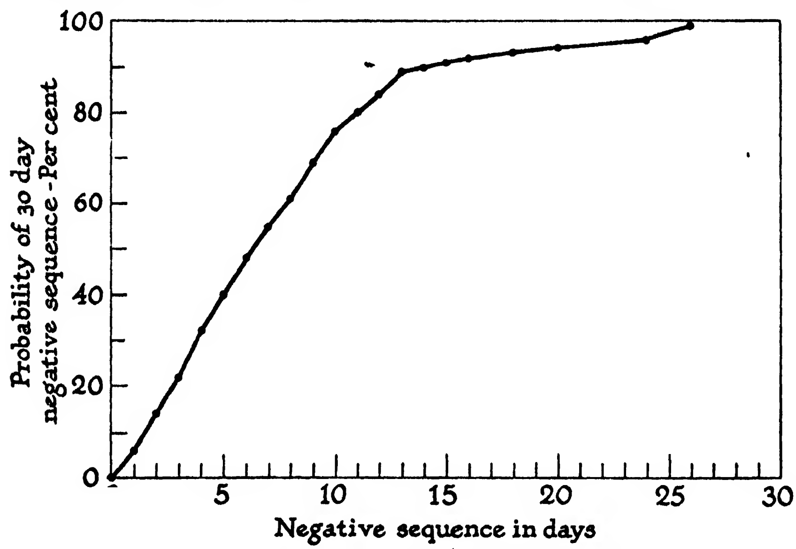

FIG. 3. A curve expressing as a percentage the probability that a single *S. ratti* infection which has been reproductively inactive for a given number of days will continue negative for a total of 30 days instead of becoming patent again. Based on 1,148 negative sequences of from 1 to 26 days' duration which were shown by 78 single *S. ratti* with adequately determined end-points.

period is not long enough to circumscribe patency in every infection. One *S. ratti*, which is still patent, was observed to yield progeny after a consecutive negative period of 32 days.

The effect of using negative sequence of less than 30 days' duration may be shown in the mean length of life of the 78 single *S. ratti* followed to good end-points. If a consecutive negative sequence of 7 days' duration had been used for determining the end of patency, the apparent mean length of life of these 78 *S. ratti* would have been 107 days. A negative sequence of 14 days would have increased the apparent mean length of life to 141 days and a negative sequence of 21 days would have increased it to 142

days. The mean length of life of the group, when based on negative sequences of 30 or more days' duration, was 149 days.

The parallelism between the increasing mean length of life with prolongation of the period of accepted negative examination and the percentage probability curve shown in Fig. 3 is very close. Both indicate that up to 14 days' negative examination, refinement of the end-point increases rapidly. After that time prolonged negative examination to 30 days produces improvement in accuracy of the end-points but at a cost of time and energy scarcely commensurate with the more accurate results. On the basis of this evidence it is clear that the patent period of an occasional *S. ratti* might be increased by using minimum negative examination periods in excess of 30 days. Such a procedure, however, would make no significant contribution to present knowledge concerning the length of life of singly established *S. ratti*. In the absence of specific interest as to exactly how long a single *S. ratti* might remain quiescent, extension of negative examination periods beyond 30 days would be essentially sterile.

DISCUSSION

No reports have appeared in the literature dealing with the length of life of single nematode parasites other than *S. ratti* (Graham, 1935, 1937). Consequently there is no direct basis for comparing the situation prevailing for *Strongyloides ratti* with that of other nematode species.

Longevity of Mass Infections of S. ratti.—Specific information relating to the longevity of mass infections of *S. ratti* is limited to the observations of Sheldon (1937a) and Sandground (1926). The former observed progeny from an infection in rats 140 days after exposure with 500 larvae; 54, 71, and 122 days after exposure of three other groups with 1000 larvae; and 123 days after exposure of still another group to 2000 larvae. Sandground obtained progeny from two mass infections of *S. ratti* over an examination period of 22 weeks. He did not indicate whether the infections were infertile after that time.

Of 83 single *S. ratti* infections in the present study (see Table 1) 44 per cent were reproductively active for more than 20 weeks, the longest period of patency observed by Sheldon in mass infections.

Rate of Loss of S. ratti *from Mass Infections*.—Sheldon (1937c) has shown that the rate of loss of worms from rats infected with 1000 *S. ratti larvae* "was essentially the same as that revealed by the daily larval output" (Cf. Sheldon, 1937a, graph 1). Inspection of the data presented by Sheldon (1937c) showed that the rate of worm loss during the first 29 days of the infections followed a straight line when plotted on arithlog paper, thus

indicating a constant rate of loss. Between the 29th and the 36th days the loss of worms was abrupt; and at the 43rd day only 4 per cent of the infection present at the 8th day still remained in the rats. In the present study 4 per cent of 83 single *S. ratti* infections remained patent for more than 55 weeks as is shown in Table 1. There is thus little question of the influence of acquired resistance in eventually accelerating an apparently constant rate of worm loss in Sheldon's mass infections of *S. ratti*.

Loss of *S. ratti* from rats may therefore be (1) random, as in the case of single larva infections, (2) at a constant rate, as in the case of Sheldon's (1937c) mass infections during the early stages, or (3) precipitous, as occurred in Sheldon's mass infections when the developing acquired resistance became manifest.

Nippostrongylus muris *versus* Strongyloides ratti *in the Rat.*—Sheldon (1937c) also stated that the rate of loss of *S. ratti* in his experiment "differed from that reported by Chandler (1932) for *Nippostrongylus muris* in rats". The greater inherent variation of Chandler's *N. muris* counts led to a poorer straight line fit than did Sheldon's *S. ratti* counts when plotted on arithlog paper. Nonetheless they deviated around a straight line and definitely suggested that the loss of *N. muris* from rats was also at a constant rate. Until the marked acquired resistance intervened to accelerate worm loss in Sheldon's *S. ratti* experiment, there was a rate of worm loss almost identical with that obtained with *N. muris* by Chandler (1932). Evidently the respective data for these two rat parasites are comparable only over the first month of observation, and their similarity of reaction in this period deserves to be emphasized.

Longevity of Parasite versus Longevity of Host.—It is recognized that the longevity of some parasites may vary with the host species into which they are introduced. Faust, Wells, Adams and Beach (1934) suggested that for *S. stercoralis* "a month's period in the dog may be comparable to a year's duration of the same strain in man". A somewhat analogous situation also prevails for hookworm. Single experimental mass infections of *Ancylostoma duodenale* and *Necator americanus* in man have been known to persist for 81 and 64 months respectively (Kendrick, 1934), while in the experience of Sarles (1929b) single mass infections of *A. caninum* persisted in dogs for only 43 to 100 weeks.

Curtis, Dunning and Bullock (1933) have shown that 8241 rats of their August strain lived an average of 14.03 ± .04 months. The inbred strain of rats used for the present *S. ratti* study was derived from this August strain. Thus the 78 single *S. ratti* with a mean reproductive length of life of 149 ± 8 days persisted on the average for more than one-third of the average life span of the host.

Duration of S. stercoralis *in Man.*—Infections of *Strongyloides stercoralis* in human hosts have been recorded which are reputedly of long duration. Leichtenstern (1899) reported an infection of 13 years' standing, Deschiens and Taillandier (1925) one of 17 years, Fülleborn (1926) one of 24 years, and Schuurmans-Stekhoven (1928) one of 13 years. Sandground (1928) stated that "it is difficult to imagine that an individual worm may have such a long life span and since the possibility of auto-infection has not been definitely ruled out, it may be that continuous auto-infection will account for the longevity of these infections".

Although Fülleborn (1926) suggested "autoinfektion" as a mode of maintenance for these infections of long duration, he visualized it as being achieved by the penetration of the larvae "in die Haut der Analgegend". This in essence is re-infection rather than internal, auto or hyperinfection in the sense that these terms are now interpreted. On the other hand Fülleborn (1926) also recognized the fact "dass eine nach Jahrzehnten währende Lebensdauer von Strongyloides im Darme auch ohne Neuinfektion sehr wohl möglich ist, wie das Beispiel der so langlebigen Filarien ja beweist!"

Hyperinfection versus Re-infection.—If there is any correlation between the longevity of a parasite and that of its autocthonous host species then the question may be asked: Is it necessary to postulate auto or hyperinfection to account for *Strongyloides* infections of seemingly long duration? In part the conclusion that auto-infection *must* play a rôle in these cases of long standing has arisen from an impression that human infection with *Strongyloides* occurs almost exclusively in tropical regions. Thus Schuurmans-Stekhoven (1926) explained an infection, presumably of 13 years' duration, by "taking into account the long period this patient had been out of the tropics" and concluded that "it is evident that autoinfection has occurred". Kreis (1932) emphasized that "*Strongyloides* exists *par excellence* in the tropical zones"; yet one can scarcely agree with his statement that "the conditions there [insofar as *Strongyloides* is concerned] are entirely different from those in the temperate regions of the world". It is but necessary to point out that *S. papillosus* is one of the most abundant parasites of sheep even in northern latitudes, and Augustine and Davey (1939) observed a natural infection of *Strongyloides canis* (possibly *S. stercoralis*) in a five weeks old puppy in Massachusetts in March, 1938. The age of the puppy, the place and the time of the year are also suggestive. While a tropical climate may have a direct as well as an indirect relationship to both the incidence and the degree of *Strongyloides* infection, biologically it cannot be looked upon as a limiting factor.

Insufficiently emphasized have been the facts that infective larvae of

Strongyloides are extremely efficient skin penetrators and that the life cycle requires only a very short time. It is significant that Spindler (1936) used for experimental purposes "pigs that had been farrowed and raised indoors under conditions which precluded extraneous infections with any helminth parasite, except Strongyloides". Mönnig (1930) found that "control lambs showed a slight Strongyloides infection which is difficult to avoid" and Seddon and McGrath (1931) used lambs raised "worm-free" except for *Strongyloides*. Stoll (1936) observed that "*Strongyloides* [*papillosus*] . . . is a contaminant established with marked ease". When lambs reared indoors at this Institute, under conditions designed to maintain them helminth-free, do show contamination, it is invariably with *S. papillosus*.*

In view of these facts it is obvious that research conditions would be necessary to preclude re-infection in the reputed cases of auto-infection with *Strongyloides*. Consequently these cases can be accepted as demonstrating neither extreme longevity of the parasites involved nor the occurrence of auto- or hyperinfection.

In the present study with single larva infections of *S. ratti* an unexcelled opportunity for the detection of hyperinfection has been afforded. A part of the eggs produced by this parasite do hatch in the intestinal tract (Cf. Sandground, 1926, p. 374) under conditions presumably as favorable for their further hyperinfective development as those encountered by the larvae of *S. stercoralis*. Yet no increase in progeny yields from these numerous single *S. ratti* infections has ever suggested that more than a *single* worm was reproductively active in any rat. These are facts which obviously bear directly on the problem and they furnish no support whatever for the idea that hyperinfection or re-infection has occurred in these studies, and by so much definitely invalidate such hypotheses.

There is a final point that emerges concerning *S. ratti*, namely, that a nematode capable on the average of surviving in its normal host for more than one-third of the mean length of life of that host should be definitely considered a long-lived parasite.

SUMMARY

1. Of 156 single *S. ratti* from an essentially pure line which were maintained in laboratory rats by serial homogonic passage, 78 were followed by 24-hour fecal collections during the late patent and post-patent period to determine accurately the length of reproductive life. These were considered to be reproductively exhausted after they had yielded no progeny

* Observation of Dr. Norman R. Stoll.

for 30 or more consecutive days. Excluding the five *S. ratti* which are still patent, the balance (73 infections) with either no end-points or poorer end-points paralleled the preceding group by showing known lengths of life as randomly distributed as those in the former group.

2. The length of reproductive life of these 78 single worm infections with adequate end-points ranged from 8 to 477 days with a mean of 149 ± 8 days.

3. When grouped for parasitic age by 5-week intervals, the length of reproductive life of 83 infections was distributed in an approximately normal manner. The median and mean length of reproductive life (respectively 128 and 149 ± 8 days) closely approached the modal value which occurred in the 5-week interval ending with the 20th week of life.

4. The loss of single established *S. ratti* from their rat host appears to occur at random in contrast to the constant rate of loss or the precipitous loss which may occur in mass infections.

5. To a very great extent the length of reproductive life of these single *S. ratti* seems unrelated to their reproductive capacity, i. e., cessation of reproduction of a single *S. ratti* may occur soon after its establishment. Long and short-lived single *S. ratti* in this line reproduce at essentially similar rates at comparable age levels.

6. The 78 single *S. ratti* for which the end of reproductive life was determined accurately lived on the average for more than one-third of the mean length of life of the strain of laboratory rats which were used as hosts. Consequently *S. ratti* as typified in these studies is considered to be long-lived.

REFERENCES

Augustine, D. L. and Davey, D. G. (1939). Observations on a natural infection with *Strongyloides* in the dog. *J. Parasit.*, 25, 117.

Chandler, A. C. (1926). The rate of loss of hookworms in the absence of re-infections. *Indian J. Med. Res.*, 13, 625.

——— (1932). Experiments on resistance of rats to superinfection with the nematode, *Nippostrongylus muris*. *Amer. J. Hyg.*, 16, 750.

Curtis, M. R., Dunning, W. F. and Bullock, F. D. (1933). Genetic factors in relation to the etiology of malignant tumors. *Amer. J. Cancer*, 17, 894.

Deschiens, A. and Taillandier, O. (1925). Présence des larves rhabditoides de *S. stercoralis* dans le liquide duodenal receuilli par tubage. *Bull. Soc. Path. Exot.*, 18, 525.

Faust, E. C., Wells, J. W., Adams, C. and Beach, T. D. (1934). Experimental studies on human and primate species of Strongyloides. III. The fecundity of Strongyloides females of the parasitic generation. *Arch. Path.*, 18, 605.

Fülleborn, F. (1926). Hautquaddeln und "Autoinfektion" bei Strongyloidesträgern. *Arch. f. Schiffs. u. Trop. Hyg.*, 30, 721.

Graham, G. L. (1935). Minimum length of life of the parasitic stage of *Strongyloides ratti*, as determined by single larva infections. *Anat. Rec.*, 61, no. 1, supp. 1, 109. (Abstract.)

——— (1936). Studies on Strongyloides. I. *S. ratti* in parasitic series, each generation in the rat established with a single homogonic larva. *Amer. J. Hyg.*, 24, 71.

——— (1937). The infections produced in rats by a single larva of *Strongyloides ratti*. *Proc. Helm. Soc. Washington*, 4, 29.

——— (1938a). Studies on Strongyloides. II. Homogonic and heterogonic progeny of the single, homogonically derived *S. ratti* parasite. *Amer. J. Hyg.*, 27, 221.

——— (1938b). Studies on Strongyloides. III. The fecundity of single *S. ratti* of homogonic origin. *J. Parasit.*, 24, 233.

——— (1939a). Studies on Strongyloides. IV. Seasonal variation in the production of heterogonic progeny by singly established *S. ratti* from a homogonically derived line. *Amer. J. Hyg.*, 30, 15.

——— (1939b). Studies on Strongyloides. V. Constitutional differences between a homogonic and a heterogonic line of *S. ratti*. *J. Parasit.*, 25, 365.

Kendrick, J. F. (1934). The length of life and the rate of loss of the hookworms, *Ancylostoma duodenale* and *Necator americanus*. *Amer. J. Trop. Med.*, 14, 363.

Kreis, H. A. (1932). Studies on the genus *Strongyloides* (Nematodes). *Amer. J. Hyg.*, 16, 450.

Leichtenstern, O. (1899). Zur Lebensgeschichte der *Anguillula intestinalis*. *Zbl. Bakt.* I. Abt. Orig., 25, 226.

Mönnig, H. O. (1930). Studies on the bionomics of the free-living stages of *Trichostrongylus* spp. and other parasitic nematodes. *16th Report of the Director of Vet. Services and Animal Industry, Union of So. Africa*, 175.

Sandground, J. H. (1926). Biological studies on the life-cycle in the genus Strongyloides Grassi, 1879. *Amer. J. Hyg.*, 6, 337.

——— (1928). Some studies on susceptibility, resistance, and acquired immunity with *Strongyloides stercoralis* (Nematoda) in dogs and cats. *Ibid.*, 8, 507.

Sarles, M. P. (1929a). Quantitative studies on the dog and cat hookworm, *Ancylostoma braziliense*, with special emphasis on age resistance. *Ibid.*, 10, 453.

——— (1929b). The length of life and rate of loss of the dog hookworm, *Ancylostoma caninum*. *Ibid.*, 10, 667.

——— (1932). Development of an acquired resistance in rabbits by repeated infection with an intestinal nematode, *Trichostrongylus calcaratus* Ransom, 1911. *J. Parasit.*, 19, 61.

Schuurmans-Stekhoven, Jr., J. H. (1928). Researches on nemas and their larvae. III. *Strongyloides stercoralis* Bavay. *Z. Parasitenk.*, 1, 231.

Scott, J. A. (1928). An experimental study of the development of *Ancylostoma caninum* in normal and abnormal hosts. *Amer. J. Hyg.*, 8, 158.

Seddon, H. R. and McGrath, T. T. (1931). Observations upon the conditions requisite for the transmission of gastro-intestinal nematodes of the sheep. *Veterinary Research Report, No. 6, Parts I and II, Department of Agriculture, New South Wales*, 40.

Sheldon, A. J. (1937a). Some experimental studies on *Strongyloides ratti*. *Amer. J. Hyg.*, 25, 39.

——— (1937b). Studies on active acquired resistance, natural and artificial, in the rat to infection with *Strongyloides ratti*. *Ibid.*, 25, 53.

——— (1937c). The rate of loss of worms (*Strongyloides ratti*) in rats. *Ibid.*, 26, 352.

Spindler, L. A. (1936). Effect of ferrous sulphate and copper sulphate on experimental infection of pigs with the nodular worm, *Oesophagostomum dentatum*. *No. Amer. Vet.*, 17, 29.

Stoll, N. R. (1936). Observations on cattle nematode infections, with a demonstration of their secondary transmission to grazing sheep. *J. Parasit.*, 22, 386.

Winfield, G. F. (1933). Quantitative experimental studies on the rat nematode *Heterakis spumosa*, Schneider, 1866. *Amer. J. Hyg.*, 17, 168.

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STUDIES ON STRONGYLOIDES

VIII. Comparison of Pure Related Lines of the Nematode, Strongyloides ratti, Including Lines in Which Gigantism Occurred

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In the life cycle of nematodes in the genus Strongyloides a somewhat unusual situation prevails: a free-living bisexual generation may be interpolated between parasitic generations.

Strongyloides ratti, represented parasitically in the intestine of the rat host only by a female, produces larvae infective for a new host by two methods. Eggs of the parasite are shed in the feces of the host, and the rhabditiform larvae which hatch from them develop in this fecal medium outside the host's body. In the direct, or homogonic, mode of development, the progeny metamorphose directly into filariform larvae which are then ready to enter a new host and develop into fertile gynomorphic parasites. In the indirect, or heterogonic, mode of development the young rhabditiform larvae develop into sexually mature, morphologically distinct, free-living males and females which then produce a crop of filariform larvae which are morphologically indistinguishable from those produced directly and which are equally capable of infecting new hosts. Thus in the heterogonic cycle an alternation of generations occurs.

When an alternation of generations (metagenesis, heterogenesis, or variations thereof) occurs in invertebrates it has generally been recognized as contributing elements either useful or essential to the organism involved. Such has not been the case concerning Strongyloides. It has been shown by Graham ('36) that under laboratory conditions S. ratti can be passed serially from rat to rat apparently indefinitely by using only a single direct-development larva to establish each new parasitic generation. The writer has thus passed one pure line of S. ratti through sixty-nine generations up to the present time. Under such circumstances an attempt to define the service performed for the species by heterogony, which under experimentation can be by-passed for long periods of time, seems appropriate.

Most investigators are agreed that the direction of larval development is

determined at the time the egg is formed by the Strongyloides parasite, but there are several recent exceptions to this point of view. Thus Faust ('36) stated that "In the older descriptions of the free-living phase of the life cycle of Strongyloides 'direct' and 'indirect' modes of development were prominently featured, although their significance was either inadequately or inaccurately appraised" and concluded that the "more recent studies provide experimental evidence which minimizes the significance of the 'direct' and 'indirect' modes of development of Strongyloides insofar as their distinctness is concerned." In part his position on this point was based on observations made on the development of Strongyloides simiae eggs in various culture media by Beach ('36) who stated that his work proved that "the mode of development of the larvae is not predetermined in the egg, as has been previously supposed, but the larvae are governed by the influences exerted upon them in their environment."

In contrast to these facts, or opinions, it has been shown that constitutionally dissimilar lines of S. ratti may be recognized. The characteristics of a heterogonically maintained line of singly established S. ratti were reported by Graham ('39b) together with the characteristics of a homogonically maintained line from which the former was readily distinguishable on the basis of relative yields of the two progeny types alone. Graham ('40a) also presented evidence that homogonically maintained lines of singly established S. ratti may likewise be distinguished on a similar basis. These results were obtained under conditions as rigidly comparable as could be devised and the dissimilarities were of a magnitude which precluded consideration of environmental influences on the young larvae as a major factor in determining their mode of development.

The present paper proposes to delineate and compare the characteristics of the pure lines derived from a single parasitic S. ratti including heretofore undescribed lines in which gigantism of the filariform larval progeny occurred.

Materials and Methods

The strain of Strongyloides ratti used in these studies was derived, as noted by Graham ('36), from three wild rats. The pooled filariform larvae thus obtained were established as a stock infection in a laboratory rat. From the infection in this animal a pure, homogonic line of singly established S. ratti was started in January, 1935, and has been continued by serial passage until the present time. This original line of single S. ratti has been designated as direct line I. The stock infection was continued by more or less regular mass passage and, as indicated by Graham ('39a), served as the

source of the free-living female, one of whose filariform larval progeny was established in a rat as the first parasitic generation of the indirect, or heterogonic, line. It is with the subsequent history of this single parasite's progeny and their serial continuation that the present paper deals.

Details of technical procedures utilized in these studies have been reported by Graham ('36, '38a and b, '39a and b, and '40a and b). In all instances rats have been exposed to only a single filariform larva of S. ratti and the pure lines thus established continued serially. In homogonically maintained lines of the parasite, i.e., direct lines I, II, and III, and giant lines I, II, and III, passage from generation to generation has been intermediated only by a filariform larva of direct development. In the heterogonic line (Graham, '39b) passage between parasitic generations was accomplished through the agency of an impregnated female of the free-living bisexual generation from which infective filariform larvae of indirect development were obtained. These were then used singly for the exposure of helminth-free laboratory rats.

Fecal collections (24-hour basis) from the rats harboring S. ratti of the three giant lines were isolated after an incubation period of 24 hours as were those from rats harboring S. ratti of the heterogonic line and homogonic lines II and III (Graham, '39b and '40a).

As in the homogonic (direct) line II (Graham, '40a) the homogonic larvae from the giant line infections were permitted to stand in tap water in 50 cc. centrifuge tubes for an additional 24 to 48 hours as a "conditioning" period during which they usually passed through their last ecdysis and reached the infective stage. Microscopic confirmation of exsheathing was a prerequisite for use of a larva for single larva infection purposes.

Once established, each patent infection was followed in detail. The 24-hour fecal output of each rat harboring a single parasite was collected over water, placed in filter-paper-lined Petri dishes, and then incubated at an optimum temperature (28°C.). These fecal "cultures" were then isolated in the Baermann apparatus, a sedimenting funnel containing warm water, and the recovered progeny concentrated by gravity in conical bottom centrifuge tubes. The daily progeny yield of each parasite was then examined under the low power of the microscope and classified as to whether they were of direct or indirect development, each type and sex in the free-living forms being enumerated exactly. These daily examinations extended from the onset of patency until 30 or more days after the last "culture" yielding progeny was obtained. Theoretically the entire progeny output of each parasite during its reproductive life was observed and classified.

The rats used were from a highly inbred strain of yellow-hooded animals

(August strain of Curtis, Dunning, and Bullock, '33) which have been used in all studies of this series referred to earlier in this section. All were fed a uniform dry mash ration.

RESULTS

Origin of Pure, Related Lines of Single S. ratti

The parent heterogonic line. As indicated in the preceding section, all of the lines of S. ratti to be herein specifically compared originated from a single parasite of heterogonic origin in rat number 561.

The passage of the parent heterogonic line through twelve serial parasitic generations is shown diagrammatically in figure 1 together with the passage of the homogonically derived lines (direct lines II and III) and homogonically derived giant lines I and II. In the heterogonic line, a free-living bisexual generation (M × F) was intercalated between each parasitic generation. In practice a 24-hour fecal culture from a rat harboring a single heterogonically derived parasite was isolated after a 24-hour incubation period and well-developed free-living females were selected. These were placed singly in feces from helminth-free rats and incubated for 72 hours. These cultures containing the single free-living females were then isolated and the indirectly derived filariform larval progeny, if present, recovered for use in exposing rats to a single larva each for the establishment of a new parasitic generation. As a rule, about one-third of the free-living females thus isolated yielded progeny, indicating that they had been impregnated prior to the original isolation.

The selection of single free-living females as a source of filariform larvae and the establishment of the parasitic generation at the minimum level, i.e., one S. ratti in a rat host, lead to assured unity of maternal ancestry in the heterogonic line. In the parasitic generations containing more than one single worm infection it cannot be unequivocally stated that the parasites are full sisters, for it is not known whether or not polyandry occurs in the free-living generation. In any event the considerable degree of inbreeding which must have resulted in the passage of such a heterogonic

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FIG. 1. Showing the serial passage of a heterogonic line of S. ratti through twelve parasitic generations with the point of origin and serial passage through the agency of homogonic larvae of direct lines II and III. The points at which abnormally large filariform larvae of indirect development occurred in the heterogonic line and the infections established with them are indicated in relation to giant lines I and II which were continued by serial homogonic passage from these two infections respectively. Male rat hosts are indicated by squares, females by circles.

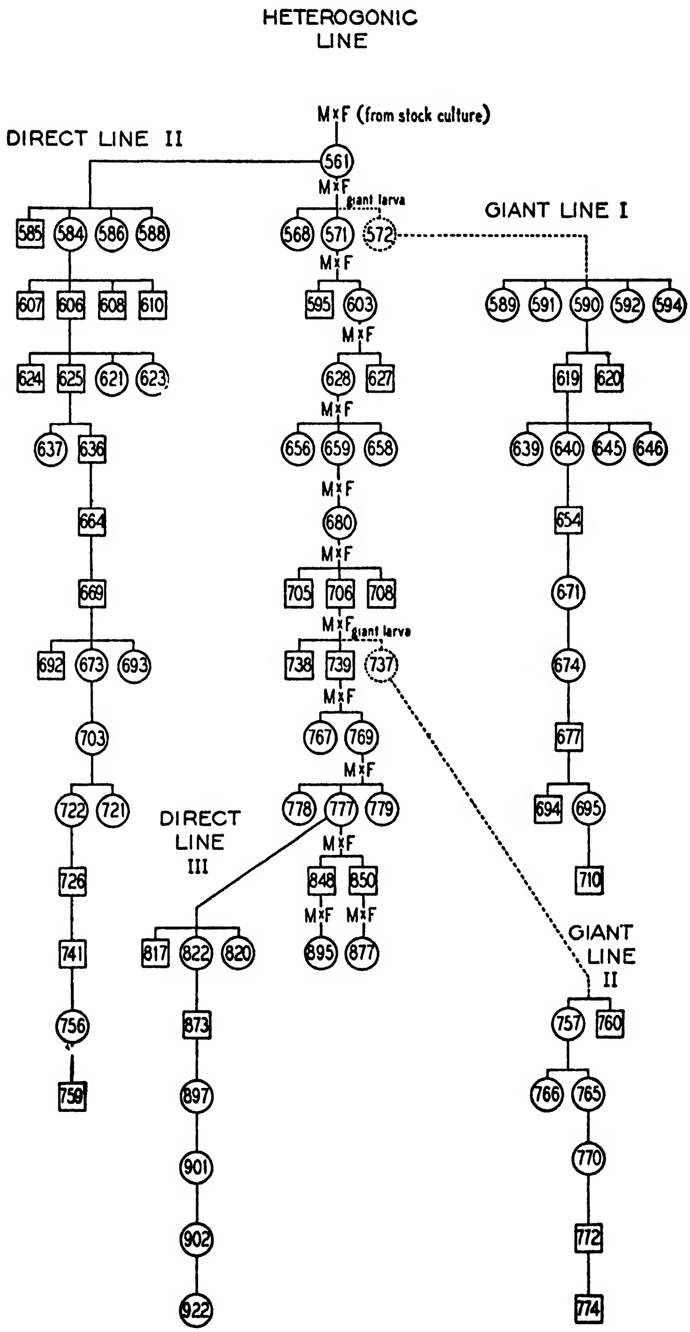
HETEROGONIC
LINE
M×F (from stock culture)
DIRECT LINE II
561
M×F
giant larva
585 584 586 588
568 571 572
GIANT LINE I
607 606 608 610
M×F
595 603
589 591 590 592 594
M×F
624 625 621 623
628 627
619 620
M×F
637 636
656 659 658
639 640 645 646
M×F
664
680
654
M×F
669
705 706 708
671
M×F giant larva
692 673 693
738 739 737
674
M×F
703
767 769
677
M×F
722 721
DIRECT
LINE
III
778 777 779
694 695
M×F
726
848 850
710
M×F M×F
GIANT
LINE
II
741
817 822 820
895 877
756
873
757 760
759
897
766 765
901
770
902
772
922
774

FIG. 1

line through twelve serial generations seems obvious. It is possible that all of the single parasitic S. ratti in the generations containing more than one infection are full sisters. The point is only of incidental interest as far as present purposes are concerned and not amenable to proof.

Direct lines II and III. After a few consecutive days of examination, it was recognized that the first generation parasite of the heterogonic line (number 561) was exhibiting characteristics, e.g., a very high percentage of free-living, adult males and females among its progeny, which clearly differentiated it from the single S. ratti in direct line I. This being true, the advisability of continuing the heterogonic line per se as well as establishing a new direct line from it was apparent.

Direct line II, established with homogonic larvae from the first generation parasite in rat number 561 in the heterogonic line, has been shown by Graham ('40a) to resemble the parent heterogonic line very closely in a number of respects, being differentiated from it principally on the basis of a considerably higher infection rate. Direct line III, established from the heterogonically derived S. ratti in rat number 777 (tenth serial generation) appeared to be similar to if not identical with direct line II, but was not sufficiently studied to justify a definitive comparison.

Recognition of biological differences in S. ratti in the heterogonic line (in comparison with single S. ratti in direct line I) had established beyond doubt the fact that constitutional deviation occurred in S. ratti. With the establishment of direct line II, it became clear that all homogonic larvae of S. ratti were not constitutionally equivalent.

Giant lines I, II, and III. When the parent heterogonic line of S. ratti was passed from the first generation (in rat number 561) to the second parasitic generation, an interesting observation was made. Two unusually large filariform larvae were observed among the progeny of the free-living female of the bisexual generation which intermediated this passage. These larvae were much larger than any of the many thousands of filariform larvae seen during the serial passage of the pure homogonic line I through many parasitic generations. Each was used for the exposure of a helminth-free laboratory rat. On the sixth day after exposure a 24-hour fecal collection was obtained from one of the exposed rats which, upon Baermann isolation, after a 24-hour incubation period, showed progeny of S. ratti to be present.

Examination of the progeny isolated on subsequent consecutive culture days indicated that the singly established parasitic female in rat number 572 from which these progeny were arising was quite distinct from her sisters

established in other rats (numbers 568 and 571) by exposure of these rats to a single, normal-sized larva each. Continued serial homogonic passage of this new line permitted the accumulation of data to a point where comparison of this "giant" line I, as well as "giant" lines II and III, with the parent heterogonic line and the other derived homogonic line (direct line II) seems desirable.

Giant line II originated from the heterogonic line through the agency of an infection (in rat number 737) established with a giant larva of indirect development which was obtained in the same manner as that used for infecting rat number 572 from which giant line I arose. This line contained only seven infections distributed in five serial generations (see fig. 1) but in all respects was like giant line I. The progeny relationships were similar; the parasites were short-lived; males of the free-living bisexual generation were observed rarely; and fecundity was low. In addition, a similar infection rate was indicated by the establishment of seven patent infections from ten exposures of rats to a single larva each.

Giant line III was established by the isolation of a giant larva (mode of development unknown) from a mixed, mass, stock infection which was developed from the two single S. ratti infections comprising the eleventh generation in the heterogonic line. It was maintained for too short a time to deserve more than passing mention, but insofar as can be judged, it conformed to the characteristics of giant lines I and II.

Variation in Length of Filariform Larvae

The abnormal size of the filariform larva of indirect development used for the establishment of the parasite (in rat number 572) from which giant line I was derived was found to be preserved in the homogonic larva produced by that initial infection. This characteristic was likewise observed in the progeny of direct development from all eighteen parasites in giant line I. Hence, this larger size of the larvae in this line is a matter of constitution and not one of chance variation.

In describing Strongyloides ratti, Sandground ('25) indicated that the filariform larvae (mode of development not specified) ranged in size from 560 μ to 736 μ with an average length of 651 μ. Some slight variation in length almost always occurs among the filariform larval progeny from a single S. ratti but even the largest larva previously noted was not comparable in length with these "giant" larvae. Several larvae from giant line I were found to measure 764 μ in length, thus being somewhat longer than the maximum length noted for filariform larvae of S. ratti by Sandground

('25), i.e., 736 μ. The smallest larva observed in a mixed, mass, stock infection derived from the pure heterogonic line measured 530 μ; the largest, a giant larva, measured 833 μ.

In figure 2, a photomicrograph of a representative direct development larva measuring 583 μ from direct line I, fifty-sixth serial generation, is shown

FIG. 2. Photomicrographs of S. ratti filariform larvae of direct development at the same magnification. The larva at the left, from the first parasitic generation in giant line III, measured 833 μ; the larva at the right, from the fifty-sixth parasitic generation in direct line I, measured 583 μ. Photomicrograph by Julian A. Carlile.

with a photomicrograph of a homogonic larva, 833 μ long, from giant line III. These two larvae were selected at random. The differential in length between so-called "giant" larvae and "normal-sized" larvae is not always as great as that shown because of normal variation, but recognition of larvae from a "giant" line never required more than a glance. Thus to the biological differences which have been shown by Graham ('39b and '40a) to exist between various pure lines of S. ratti, a bona fide morphological difference may now be added in another constitutionally distinct line of the parasite.

Comparison of Biological Characteristics in the Giant Lines, the Parent Heterogonic Line, and the Related Direct Line II

The characteristics which distinguish the heterogonic line from direct line I were presented in detail by Graham ('36b) with the object of demonstrating constitutional dissimilarity in two pure lines of S. ratti. The characteristics distinguishing direct line II from direct line I were noted in similar detail by Graham ('40a) in order to demonstrate that the potentialities of homogonic larvae in pure lines of S. ratti may differ. Since the outstanding features of the parent heterogonic line and the derived direct line II are on record, primary attention will herein be devoted to defining the characteristics of giant line I in its relationship to these closely related lines.

In addition to the consistently larger size of the filariform larvae in the giant lines, other critical points of difference involved (1) the relative yields of direct and indirect progeny, (2) fecundity or reproductive rate, (3) length of reproductive life, (4) frequency of production of free-living males, and (5) the infection rate.

Relative yields of direct and indirect progeny. The relationship existing between direct and indirect progeny from the eighteen single S. ratti in giant line I is shown in figure 3 as average daily progeny by weeks of parasitic age. Although adults of indirect development were slightly more numerous than larvae of direct development (52%), during the early weeks of the infections the predominant production of direct development larvae made the relationship of the two progeny types appear somewhat like that in direct line I (Graham, '38a, fig. 2). In the later weeks of the infections the predominant production of indirect adults makes the curves resemble those from the heterogonic line (Graham, '39b, fig. 2) and the homogonic, direct line II (Graham, '40a, fig. 1). Actually, the progeny relationships in this giant line seem intermediate between the extremes previously recognized in other lines.

The reproductive rate (fecundity). The lower reproductive rate observed in the S. ratti from giant line I in comparison with those of the parent heterogonic line and direct line II is shown in figure 4 by means of cumulative progeny yield curves. The weekly increment is based on the average progeny output from patent culture days. The parallelism of the curves for direct line II and the parent heterogonic line stands in marked contrast to the low reproductive rate in giant line I.

Of the eighteen S. ratti in giant line I, only one produced more than 300 offspring during its period of reproductive life. One infection which re-

mained patent into the fifth week produced only seventeen progeny. The least fecund S. ratti in the heterogonic line produced more than 400 progeny,

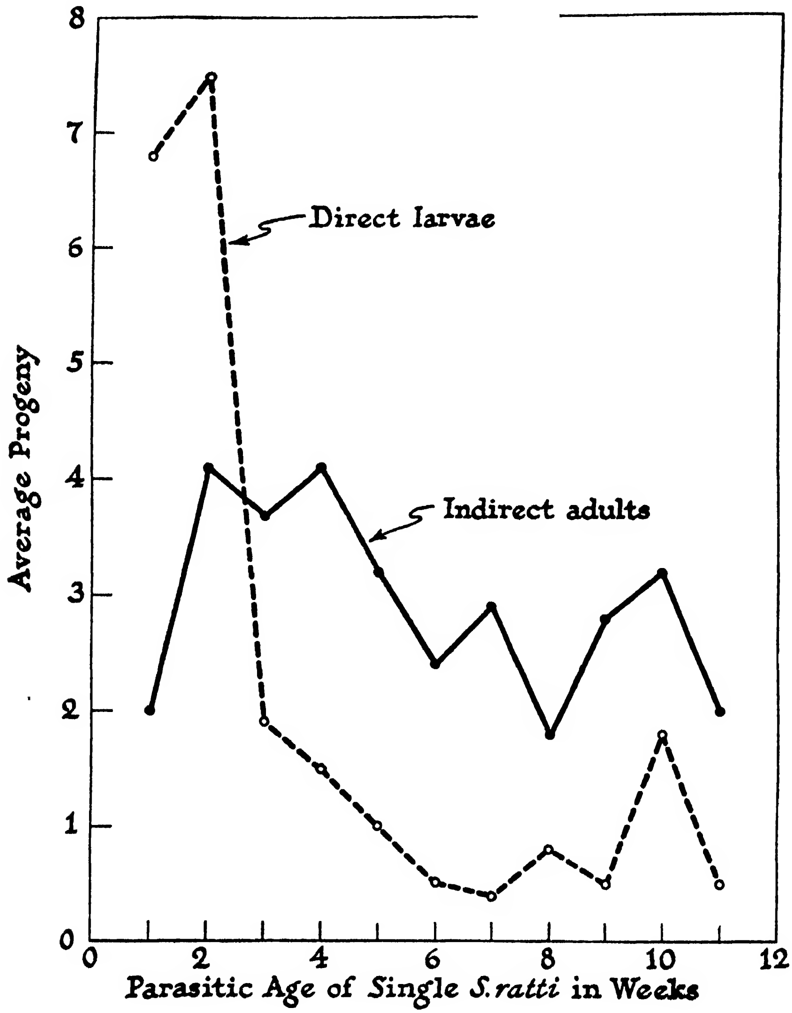

FIG. 3. Average numbers of free-living adult and direct development larvae in the progeny secured from eighteen single S. ratti parasites in the homogonically passed giant line I. Observations made on progeny in cultures obtained from 24-hour fecal collections from the rat host.

and three yielded more than 2,100 offspring each. S. ratti in direct line II, being longer-lived than those in the parent heterogonic line, produced a correspondingly larger number of progeny; those in rat numbers 586 and 608 produced in excess of 3,000.

Length of reproductive life. The eighteen infections in giant line I had a mean reproductive life span of only 39 ± 3 days in comparison with life

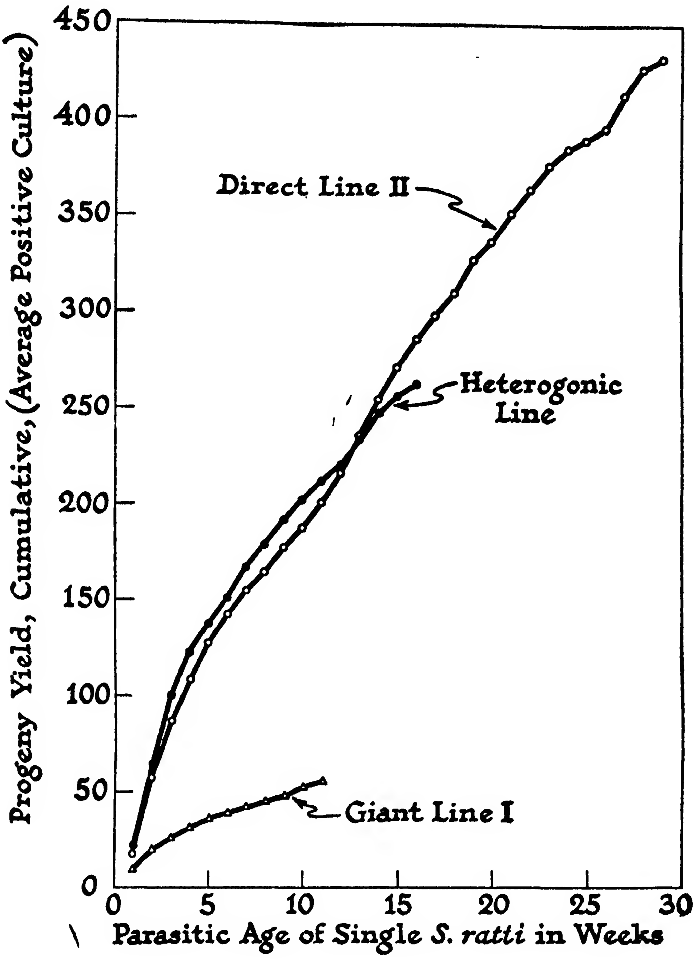

FIG. 4. The cumulative progeny yield from 25 S. ratti of the heterogonic (indirect) line, 26 S. ratti in the derived direct line II, and eighteen S. ratti in the derived giant line I. Weekly accumulations for each curve are based on the average progeny yields of positive cultures. In the heterogonic line 1,229 culture days yielded 24,932 progeny; in direct line II, 1,798 culture days yielded 33,654 progeny; and in giant line I, 435 culture days yielded 2,880 progeny.

spans of 72 ± 4 and 90 ± 8 days in the parent heterogonic line and direct line II respectively, the difference from both being statistically significant. In contrast to all of these Graham ('40b) has reported a mean reproductive length of life of 149 ± 8 days for singly established S. ratti in another homo-

gonically maintained line, i.e., direct line I. In giant line I, as well as in giant lines II and III, the short length of life and the concentrated production of larvae of direct development during the first few weeks of parasitic existence necessitated frequent passage to maintain the lines. Giant lines I and II were lost because of failure to multiply infections at each successive generation. In maintaining direct line I, now in its sixty-ninth serial generation, intervals of as long as 3 months between passages have not jeopardized its serial maintenance.

Frequency of production of free-living males. The infrequent occurrence of free-living males among the progeny from the heterogonically derived

TABLE 1

Comparison of Progeny Relationships and Other Distinguishing Criteria of the Parent Heterogonic Line of Strongyloides ratti with Those of the Derived Homogonic Lines, Direct Line II and Giant Line I

	Direct Line II	Heterogonic Line	Giant Line I
No. of parasites	26	25	18
Serial generation	1–13	1–12	1–9
No. of cultures with one or more offspring	1,798	1,229	435
Progeny			
Direct larvae	2,900	2,530	1,393
Indirect males	7,567	6,102	20
Indirect females	23,187	16,300	1,467
Total progeny	33,654	24,932	2,880
Percentage of indirect development	91.4%	89.9%	51.6%
Ratio (approximate) of males to females	1:3	1:3	1:75
Infection rate	74%	39%	75%
Mean length of life in days	90 ± 8	72 ± 4	39 ± 3

S. ratti in rat number 572 (see fig. 1) as well as from all eighteen S. ratti in giant line I necessitated passage of the line by means of homogonic larvae. When males were observed in cultures, the females were isolated singly in feces from helminth-free rats, but no larvae of indirect development were ever obtained. While detailed study of the few males obtained was not made, they were observed with some care and almost all appeared abnormal. The reproductive organs were not developed in the manner observed in the males from the heterogonic line and direct line II nor were they distended proximally with sperm as was common in young adult males in the other lines of S. ratti. In a few instances the spicules and gubernaculum were poorly sclerotized and showed very faintly in outline.

The relationships between the three lines of S. ratti under comparison are shown in table 1. While the ratio of one male to approximately

seventy-five females in the bisexual generation in giant line I was somewhat lower than the approximate ratio of 1 to 200-300 observed in direct line I (Graham, '40a, tables 1 and 2), it is practically at the same level as the ratio in direct line I when considered in conjunction with the ratios of 1 to 3 which were obtained in the heterogonic line and direct line II. However, the males which occur in direct line I on rather rare occasions are known to be fertile, for frequently the females which they usually accompany have shed eggs which were in the process of development and from which rhabditiform larvae hatched. Thus it seems probable that the free-living males of the giant lines are sterile, although further study might reveal the occurrence of fertile males.

The infection rate. The infection rate in giant line I (75%) appears to be identical with that in the related direct line II. This is the only point of similarity which seems to exist between these two closely related lines of singly established S. ratti. Both differ in this respect from the parent line from which they originated (see table 1). In direct line I, an infection rate of 26.2% has been observed (Graham, '39b).

DISCUSSION

The differences shown by Graham ('39b and '40a) together with those reported in this paper indicate clearly that constitutionally distinct lines of S. ratti may be recognized. Biological dissimilarities as well as a distinct morphological difference have been observed in comparing single S. ratti in four pure, serial lines, i.e., an independent direct line I and three closely related lines: a heterogonic line, direct line II, and giant line I. Other collateral lines, namely, direct line III and giant lines II and III, likewise showed the major characteristics of their comparable homologous lines (direct line II and giant line I) although they were less thoroughly studied.

The constancy of characteristics exhibited by the homogonically maintained lines and the fact that distinctive new lines of S. ratti were derived from the heterogonic line by way of heterogony seems significant. They obviously suggest that the answers may be at hand for the questions asked by Graham ('36) as to what rôle "may heterogony play in the maintenance of parasitic fertility and is dioecism an essential element of Strongyloides biology?" Rather than being a mere reversion to the ancestral heterogony of the genus as suggested by Sandground ('26), heterogony may be the vital agency whereby the species is renewed and rejuvenated. It is conceivable that the survival of S. ratti as a species may actually depend on heterogony, for it is clear that a short parasitic life span, a low fecundity, a low infection rate, and the absence of the enormous multiplicative factor involved in the free-living phase of heterogonic reproduction, as well as the inability

of the infective larvae to survive long in nature, are all distinct handicaps to a parasitic species.

May not the resiliency and apparent ubiquity of S. ratti (and perhaps other species of Strongyloides) in nature be dependent on the capacity of the species to adapt itself rapidly and adequately to new environmental conditions which even in the parasitic phase (Graham, '39a) seem not to be constant? Heterogony appears to be a biological mechanism capable of performing these important functions in the case of S. ratti. In this connection the observation of parthenogenetic development of eggs in parasitic S. ratti females by Chitwood and Graham ('40) seems significant. Parthenogenesis in the parasitic phase of the S. ratti life cycle may thus explain the constancy of biological characteristics in homogonically maintained lines of the parasite. Under such conditions it is not difficult to understand the importance of the part played by the free-living bisexual generation in the economy of this parasite.

Concerning the unusual size of the filariform larvae in giant lines I, II, and III it is but necessary to note that gigantism is a constantly recurring biological phenomenon. Its occurrence in a nematode is therefore not particularly remarkable, although it is distinctive as far as parasitic nematodes of vertebrates are concerned because it is definitely established on a constitutional basis. In the present case it is not unreasonable to suppose that it may be the result of polyploidy. Such an explanation might find further support in the abnormalities observed in the free-living males of the heterogonic generation as well as in the apparent sterility of these males. No effort has been made to analyze the problem on a cytological basis nor have any attempts been made to crossbreed free-living adults from any of these pure lines. Both are attractive problems, but a considerable degree of technical skill would be necessary, due to the small size of the nematodes.

The "giant" larvae were frequently accompanied by free-living females of unusual size but these may have an analogy in the facultatively parasitic nematode, Neoaplectana glaseri, which may, as McCoy, Girth, and Glaser ('38) have shown, produce giant females under certain conditions. They suggested that the giant forms of N. glaseri may be produced under conditions of very low parasite density, abundant food supply, and late fecundation of the females due to scarcity of males thus permitting excessive somatic growth. It seems possible that in nonfecundated free-living S. ratti females excessive size may be due predominantly, if not exclusively, to rapid somatic growth rather than to constitutional factors. In the "giant" lines of S. ratti as well as in direct line I in which free-living males were rare, many of the free-living females were much larger than those observed in direct lines

II and III and the parent heterogonic line in which males were numerous and a high percentage of the females were fecundated. This suggests that the larger size of these females may be due to nutritional factors, energy being consumed in somatic growth rather than being expended in egg production, although some nonviable eggs are usually produced by unimpregnated females.

Among many parasitic nematodes it is known that large infections tend to a very great extent to be composed of worms distinctly smaller than those obtained in small or medium-sized infections. This variation in size may also have a nutritive basis, possibly conditioned by immunological factors.

Females of the parasitic generation in the three giant lines were not examined to determine whether or not they also showed the gigantism which characterized the filariform larvae from which they developed. This point must await the establishment of a "giant" strain at a mass infection level to provide a plentiful supply of the parasites.

SUMMARY

1. In passing S. ratti serially by means of single larva infections with filariform larvae of indirect development, larval progeny were obtained from single, free-living females on two occasions which were approximately one-third to one-half larger than normal-sized larvae from the same worms. Single larva infections with these "giant" larvae indicated that the parasitic females which developed from them were constitutionally different from their sister parasites.

2. Serial passage from these two parasites was by homogonic passage in giant lines I and II respectively. The characteristics exhibited by eighteen single S. ratti in giant line I (passed through nine parasitic generations) were distinct from those of S. ratti in direct lines II and III which were also derived homogonically from the same heterogonic line.

3. S. ratti in giant line I produced approximately equal numbers of direct larval progeny and free-living bisexual adults. Only a small percentage of the free-living adults were males and these appeared to be infertile.

4. In addition to producing only direct development larvae of abnormal size, S. ratti in the giant lines were very short-lived and showed a low reproductive rate, although the infection rate in giant lines I and II was very high, being comparable to that in the closely related direct line II.

5. Comparison of these various pure lines of singly established S. ratti provides ample evidence that homogonic larvae of this parasite may be constitutionally dissimilar. The accumulated evidence from the study of these lines indicates that new lines arise through the agency of heterogony.

Heterogony appears to be an essential biological process involved in the maintenance of the species and not merely an evolutionary vestige of ancestral type.

LITERATURE CITED

Beach, T. D. 1936 Experimental studies on human and primate species of Strongyloides. V. The free-living phase of the life cycle. Am. J. Hyg., vol. 23, pp. 243–277.

Chitwood, B. G., and G. L. Graham 1940 Absence of vitelline membranes on developing eggs in parasitic females of Strongyloides ratti. J. Parasitol., vol. 26, pp. 183–190.

Curtis, M. R., W. F. Dunning, and F. D. Bullock 1933 Genetic factors in relation to the etiology of malignant tumors. Am. J. Cancer, vol. 17, pp. 894–923.

Faust, E. C. 1936 Strongyloides and strongyloidiasis. Rev. Parasitol., vol. 2, pp. 315–341.

Graham, George L. 1936 Studies on Strongyloides. I. S. ratti in parasitic series, each generation in the rat established with a single homogonic larva. Am. J. Hyg., vol. 24, pp. 71–87.

——— 1938a Studies on Strongyloides. II. Homogonic and heterogonic progeny of the single, homogonically derived S. ratti parasite. Am. J. Hyg., vol. 27, pp. 221–234.

——— 1938b Studies on Strongyloides. III. The fecundity of single S. ratti of homogonic origin. J. Parasitol., vol. 24, pp. 233–243.

——— 1939a Studies on Strongyloides. IV. Seasonal variation in the production of heterogonic progeny by singly established S. ratti from a homogonically derived line. Am. J. Hyg., vol. 30 (sect. D), pp. 15–27.

——— 1939b Studies on Strongyloides. V. Constitutional differences between a homogonic and a heterogonic line of S. ratti. J. Parasitol., vol. 25, pp. 365–375.

——— 1940a Studies on Strongyloides. VI. Comparison of two homogonic lines of singly established S. ratti. J. Parasitol., vol. 26, pp. 207–218.

——— 1940b Studies on Strongyloides. VII. Length of reproductive life in a homogonic line of singly established S. ratti. Rev. Med. Trop. y Parasitol., Bact., Clin. y Lab., Habana (in press).

McCoy, E. E., H. B. Girth, and R. W. Glaser 1938 Notes on a giant form of the nematode Neoaplectana glaseri. J. Parasitol., vol. 24, pp. 471–472.

Sandground, J. H. 1925 Speciation and specificity in the nematode genus Strongyloides. J. Parasitol., vol. 12, pp. 59–80.

——— 1926 Biological studies on the life-cycle in the genus Strongyloides Grassi, 1879. Am. J. Hyg., vol. 6, pp. 337–388.

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THERMAL INACTIVATION RATES OF FOUR PLANT VIRUSES

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Tobacco-mosaic virus (*Marmor tabaci* H. var. *vulgare* H.)* and tobacco-necrosis virus (*M. lethale* H.) have about the same 10-minute thermal inactivation point, ca. 92°C.[10, 11] On the other hand, they react to aging *in vitro* at room temperature quite differently from each other; tobacco-mosaic virus remains active for years when stored at room temperature but tobacco-necrosis virus becomes inactive after about 20 days under the same conditions.[14] These facts suggested that the two viruses might have different temperature coefficients of inactivation. Studies were, therefore, undertaken to investigate this possibility. In a preliminary report[12], it was stated that the temperature coefficient, $Q_{10°}$, of tobacco-necrosis virus is about 4, whereas that of tobacco-mosaic virus is of the order of 750. Actually, as in chemical reactions generally, $Q_{10°}$ is not a constant but varies with temperature so that the above figures represent averages for temperatures between 70° and 95°C. Because of the striking difference observed between the inactivation rates for tobacco-mosaic and tobacco-necrosis viruses, it seemed worth while to compare them with the inactivation rates of other plant viruses. Data on alfalfa-mosaic virus (*M. medicaginis* H.) and tobacco-ringspot virus (*Annulus tabaci* H. var. *virginiensis* H.) both of which have a thermal inactivation point of about 65°C.[2, 5, 9, 13], are, therefore, included in the present paper.

Materials and Methods

All four of the viruses used in this investigation were propagated in greenhouse-grown Turkish tobacco (*Nicotiana tabacum* L.) plants in 6-inch pots. Leaves bearing primary lesions were used as a source of tobacco-necrosis virus and systemically infected leaves as a source of the other 3 viruses. Diseased leaves were ground in a meat grinder and juice was extracted from them by passage through a single thickness of cheese-cloth. Tightly corked 7 × 70 mm. test tubes each containing 1 c.cm. of the juice were heated by immersing them completely in water that was constantly

* Latin binomials used in this paper are taken from the Handbook of Phytopathogenic Viruses.[8]

stirred and held within ±0,2°C. of the desired temperature by means of a thermostatically controlled electric heating element. Immediately after the heat treatment, the tubes were plunged into cold water and as soon as possible thereafter the juice was used to inoculate test plants.

The test plants used in the present study respond to infection by the development of necrotic lesions at the site of inoculation. It has been shown in previous work with the viruses of tobacco mosaic[13] and tobacco necrosis[11] that, over a considerable range in virus concentration, the logarithm of the numbers of lesions produced by these viruses is a linear function of the logarithm of the virus concentration. The slope of the straight line fitted to such data approximates, but is usually somewhat less than, unity. In the present study, it has been assumed that the virus concentration of a sample is equivalent to the number of lesions produced by the sample. The effect of this assumption on the calculated rates of virus inactivation will be discussed later.

The Black variety of cowpea (*Vigna sinensis* Endl.) was used as a test plant for tobacco-necrosis and tobacco-ringspot viruses, the Early Golden Cluster variety of beans (*Phaseolus vulgaris* L.) for alfalfa-mosaic virus, and *Nicotiana glutinosa* L. for tobacco-mosaic virus. The two primary leaves on each of 16 cowpea plants or on each of 8 bean plants were inoculated with a single virus sample. *N. glutinosa* plants were trimmed to 5 leaves each and 2 such plants were used for determining the concentration of a single tobacco-mosaic virus sample. Inoculations were made by rubbing the upper surface of test-plant leaves with a cheese-cloth pad which had been soaked with the virus sample to be tested.

Hydrogen-ion concentration determinations were made at room temperature by means of a glass electrode.

EXPERIMENTAL

Inactivation data. The lesion counts for heated and unheated samples of tobacco-necrosis virus are summarized in table 1; those for alfalfa-mosaic virus are given in table 2; and those for tobacco-ringspot virus in table 3. Table 4 summarizes inactivation data obtained at temperatures of 92,5°C. and 85°C., respectively, with a mixture of equal volumes of juice from tobacco plants affected by tobacco necrosis and juice from tobacco plants affected by tobacco mosaic. The k values given in the tables are first order reaction velocity constants. The calculation of these constants is discussed below.

The velocity constant. In order to calculate the inactivation rates of viruses from the data given in the tables it is necessary to know whether

inactivation is a 0, a 1st, or a 2nd order reaction. A 0 order reaction progresses at a constant rate regardless of the concentration of the reacting material, as in a surface reaction. In a 1st order reaction the number of particles inactivated at any given instant is directly proportional to the total number of particles of the reactant at that instant. In a 2nd order reaction it is necessary for 2 particles to come together and react before they can be inactivated; the rate of inactivation is thus directly proportional to the square of the concentration at any given instant. Equations for the 3 types of reactions may be written in the following form:

$$\text{0 order } C = C_0 - k_0\tau \qquad \text{(Eq. 1)}$$
$$\text{1st order } \log_e C = \log_e C_0 - k_1\tau \qquad \text{(Eq. 2)}$$
$$\text{2nd order } 1/C = 1/C_0 + k_2\tau \qquad \text{(Eq. 3)}$$

where C_0 is the initial virus concentration, C is the virus concentration at time τ, and k is the velocity constant of inactivation.

The data of tables 1, 2, and 3, as well as data previously published for tobacco-mosaic virus[10], were examined to determine whether virus inactivation follows a 0, a 1st, or a 2nd order reaction. In those experiments in which inactivation of the virus had proceeded only to about 50 to 60 per cent completion, as in the experiment with tobacco-ringspot virus at 45°C. (table 3), it was possible to fit straight lines to the data when either C, $\log_e C$, or $1/C$ was plotted against τ. This merely indicates that the variation in the lesion count method is sufficient to interfere with determination of the order of the reaction when only the range from 0 to 60 per cent inactivation is studied. In the other experiments, when $\log_e C$ was plotted against τ for inactivation of the viruses at various temperatures, the points obtained were found to fit straight lines, and when C and $1/C$ were plotted against τ, the points fell along curved lines. It is, therefore, concluded that under the conditions of the experiments, thermal inactivation of the 4 viruses studied followed the course of a first order reaction. It is of interest in this connection that heat inactivation of antistaphylococcus bacteriophage likewise follows the course of a first order reaction[6].

The velocity constants of inactivation of each virus at various temperatures were calculated by the method of least squares according to equation 2 from the data given in tables 1, 2, 3, and 4. These k values are summarized in the tables. The constants for tobacco-mosaic virus were calculated in the same manner from previously published data[10] and are given in table 5. It was necessary to estimate the constants at 95°C. and 92°C. for tobacco-mosaic virus in undiluted juice and the constants at 91°C. and 87°C. for the diluted virus from the 10-minute survival values recorded in tables 1, 2, 3, 8, and 9 of the paper referred to above.

In figure 1 the inactivation data for tobacco-mosaic virus at 90°C., for tobacco-necrosis virus at 86°C., for alfalfa-mosaic virus at 62,5°C., and for tobacco-ringspot virus at 50°C. were averaged from the various tests

TABLE 1

Data on Thermal Inactivation of Tobacco-Necrosis Virus at Several Different Temperatures

Temperature degrees C.	p_H room temperature	Lesions in 32 cowpea leaves. Time in minutes								k first order min.$^{-1}$	
		0	1	2	3	4	5	6	7		
	5,6	4 250	444	85	10	0	0	0	0	1,98	
95	5,86	16 000	2887	285	38	3	1	0	0	2,03	2,12
	5,6	14 400	2114	254	40	6	0	0	0	1,95	
		0	2	4	6	8	10	12	14		
	—	4 900	880	294	35	6	3	1	0	0,728	
90	5,6	5 615	214	121	65	4	0	0	0	1,010	0,793
	—	5 600	516	193	26	6	1	0	0	0,836	
		0	3	6	9	12	15	18	21		
	—	8 400	1070	328	58	20	4	6	18	0,330	
86	—	4 900	585	249	37	11	5	1	1	0,414	0,449
	5,6	11 200	1626	344	108	15	7	0	0	0,496	
		0	7	14	21	28	35	42	49		
	5,6	5 930	139	30	5	6	0	0	0	0,245	
80	—	8 000	1588	486	111	48	8	3	—	0,186	0,166
	5,6	12 800	1726	1080	476	103	23	14	11	0,147	
		0	15	30	45	60	75	90	105		
	5,58	4 900	1227	238	79	21	1	1	1	0,0895	
75	5,62	7 700	1540	122	77	15	4	3	0	0,0610	0,095
	5,85	7 000	1687	292	70	15	5	1	2	0,0857	
		0	30	60	90	120	150	180	210		
	5,7	5 320	223	33	5	1	3	1	0	0,0450	
70	6,4	8 000	375	68	8	7	0	0	0	0,0598	0,0496
	5,7	16 000	1059	165	53	15	6	0	0	0,0508	

at each temperature and plotted with $\log_e C$ as a function of τ. The slopes of the straight lines fitted to the points thus obtained are the velocity constants of inactivation of the viruses at the temperature specified.

Thornberry, Valleau, and *Johnson*[17] have published data on thermal inactivation of tobacco-mosaic virus in dried air-cured White Burley tobacco at a series of temperatures. From the data in their table 1 it was possible to

calculate the velocity constant of inactivation at 9 different temperatures, using equation 2 and the method of least squares. These velocity constants are given in table 6.

Factors affecting the estimation of k. The variation in the velocity constants of inactivation for a given virus at any particular temperature may

TABLE 2

Data on Thermal Inactivation of Alfalfa-Mosaic Virus at Several Different Temperatures

Temperature degrees C.	pH room temperature	Lesions in 16 bean leaves. Time in minutes								k first order min.⁻¹	
		0	0,5	1,0	1,5	2,0	2,5	3,0	3,5		
62,5	5,77	2235	1776	775	505	80	55	29	5	1,74	0,89
	5,68	2663	1252	741	458	526	432	424	189	0,605	
		0	2	4	6	8	10	12	14		
	5,49	2799	664	264	250	117	152	53	30	0,279	
60	5,83	4594	1555	937	298	58	17	1	1	0,652	0,52
	5,72	2675	352	128	29	13	2	0	0	0,677	
		0	4	8	12	16	20	24	28		
57,5	5,80	3417	1119	849	421	60	29	4	1	0,286	0,21
	5,68	2204	827	532	168	86	9	13	30	0,190	
		0	5	10	15	20	25	30	35		
	5,49	1885	513	664	221	293	179	182	166	0,062	
55	5,72	3144	1030	541	712	415	207	65	244	0,084	0,065
	5,89	5850	2129	3918	1229	902	1089	511	885	0,058	
		0	20	40	60	80	100	120	140		
52,5	5,75	2162	636	294	89	49	5	11	2	0,049	0,042
	5,75	6012	2891	181	67	91	2	104	38	0,039	
		0	30	60	90	120	150	180	210		
50	5,60	1381	1145	774	441	609	247	72	152	0,013	0,013
	5,76	2339	1094	722	515	189	149	236	178	0,013	

be seen by comparison of the *k* values for the different tests in any one experiment. There may be several factors responsible for this variation. It is known that the greatest accuracy to be expected in estimation of virus concentration from lesion counts on the number of test plant leaves used in these tests is of the order of 20 per cent.[8] The inability to estimate virus concentration more accurately than this probably is responsible for some of the variation in calculated *k* values.

Another source of error in estimation of inactivation rates is a consequence of the previously mentioned assumption that virus concentration is equiva-

TABLE 3

Data on Thermal Inactivation of Tobacco-Ringspot Virus at Several Different Temperatures

Temperature degrees C.	pH room temperature	Lesions in 32 cowpea leaves. Time in minutes								k first order min.$^{-1}$	
		0	1	2	3	4	5	6	7		
65	5,54	6746	1744	769	394	338	174	374	172	0,453	0,48
	5,42	1003	495	197	115	85	45	50	25	0,501	
		0	2	4	6	8	10	12	14		
60	5,60	8295	3049	1920	1084	579	250	290	184	0,269	0,26
	5,88	5748	1600	675	371	523	227	232	98	0,244	
		0	5	10	15	20	25	30	35		
55	5,96	8000	2337	694	211	136	31	41	9	0,185	0,091
	5,88	5740	2791	1796	1265	1090	874	538	321	0,073	
		0	10	20	30	40	50	60	70		
50	5,94	3312	4312	2836	2283	1920	1621	1192	1238	0,018	0,015
	5,61	4850	3982	4033	2308	1971	2444	1983	2311	0,012	
		0	30	60	90	120	150	180	210		
45	5,77	5005	4494	3191	3608	2792	3190	3966	2969	0,0018	0,0022
	5,55	1585	1900	1233	1479	1302	1425	1341	759	0,0026	

TABLE 4

Numbers of Lesions Produced by Inoculation with a Mixture of Tobacco-Mosaic and Tobacco-Necrosis Viruses Heated for Various Periods of Time at 92,5°C. and 85°C., Respectively

Temperature degrees C.	Virus	Test No.	Lesions in 32 cowpea leaves. Time in minutes								k first order min.$^{-1}$	
			0	1	2	3	4	5	6	7		
92,5	Mosaic	1	5 100	1950	320	7	0	0	0	0	2,2	2,3
		2	4 800	755	328	12	1	0	0	0	3,5	
			0	1	2	3	4	5	6	7		
	Necros.	1	6 400	2062	828	372	106	64	17	13	1,0	1,0
		2	8 000	1897	898	262	124	61	8	1	1,3	
		3	12 800	5400	1568	619	237	69	38	17	1,1	
			0	45	90	135	180	225	270	315		
85	Mosaic	1	8 000	5600	4237	3558	1868	1348	876	325	$9,5 \times 10^{-3}$	$8,4 \times 10^{-3}$
		2	16 850	9800	6593	5394	2834	2554	2158	1390	$7,6 \times 10^{-3}$	
			0	3	6	9	12	15	18	21		
	Necros.	1	9 600	3763	853	473	153	68	23	9	$3,3 \times 10^{-1}$	$3,3 \times 10^{-1}$
		2	3 200	1188	358	214	48	29	14	0	$3,1 \times 10^{-1}$	

TABLE 5

Calculated Velocity Constants of Inactivation of Tobacco-Mosaic Virus at Different Temperatures

Virus	Test	Temperature degrees C.								
		95 min.$^{-1}$	92 min.$^{-1}$	91 min.$^{-1}$	90 min.$^{-1}$	87 min.$^{-1}$	85 min.$^{-1}$	80 min.$^{-1}$	75 min.$^{-1}$	68 min.$^{-1}$
Undiluted	1				$1,6 \times 10^{-1}$		$3,9 \times 10^{-3}$	$6,1 \times 10^{-4}$	$1,5 \times 10^{-4}$	$9,5 \times 10^{-5}$
	2				$2,9 \times 10^{-1}$		$5,4 \times 10^{-3}$	$5,4 \times 10^{-4}$	$1,5 \times 10^{-4}$	$9,0 \times 10^{-5}$
	3				$1,1 \times 10^{-1}$		$4,3 \times 10^{-3}$	$5,6 \times 10^{-4}$	$7,6 \times 10^{-5}$	$8,6 \times 10^{-5}$
	4							$5,2 \times 10^{-4}$		$5,6 \times 10^{-5}$
	5							$8,4 \times 10^{-4}$		$9,0 \times 10^{-5}$
	Mean	6,9*	$8,5 \times 10^{-1}$*		$1,2 \times 10^{-1}$		$4,5 \times 10^{-3}$	$5,3 \times 10^{-4}$	$1,5 \times 10^{-4}$	$8,4 \times 10^{-5}$
Diluted 1:20	1						$1,9 \times 10^{-1}$	$1,3 \times 10^{-2}$	$2,5 \times 10^{-3}$	$2,8 \times 10^{-4}$
	2						$1,6 \times 10^{-1}$	$8,6 \times 10^{-3}$	$2,3 \times 10^{-3}$	$2,4 \times 10^{-4}$
	3						$1,3 \times 10^{-1}$	$8,8 \times 10^{-3}$	$1,6 \times 10^{-3}$	$1,6 \times 10^{-4}$
	4						$1,2 \times 10^{-1}$	$6,3 \times 10^{-3}$	$3,3 \times 10^{-3}$	
	Mean			6,6*		$4,1 \times 10^{-1}$*	$1,3 \times 10^{-1}$	$9,4 \times 10^{-3}$	$2,2 \times 10^{-3}$	$2,1 \times 10^{-4}$

* Estimated.

lent to lesion counts. This implies that a straight line with a slope of unity would be obtained when the numbers of lesions produced by the virus samples are plotted against the concentration of the samples. Although the exact relationship between numbers of lesions produced by the virus samples used in the present study and the concentration of these samples is unknown, it is probable, in the light of past experience, that the slope of

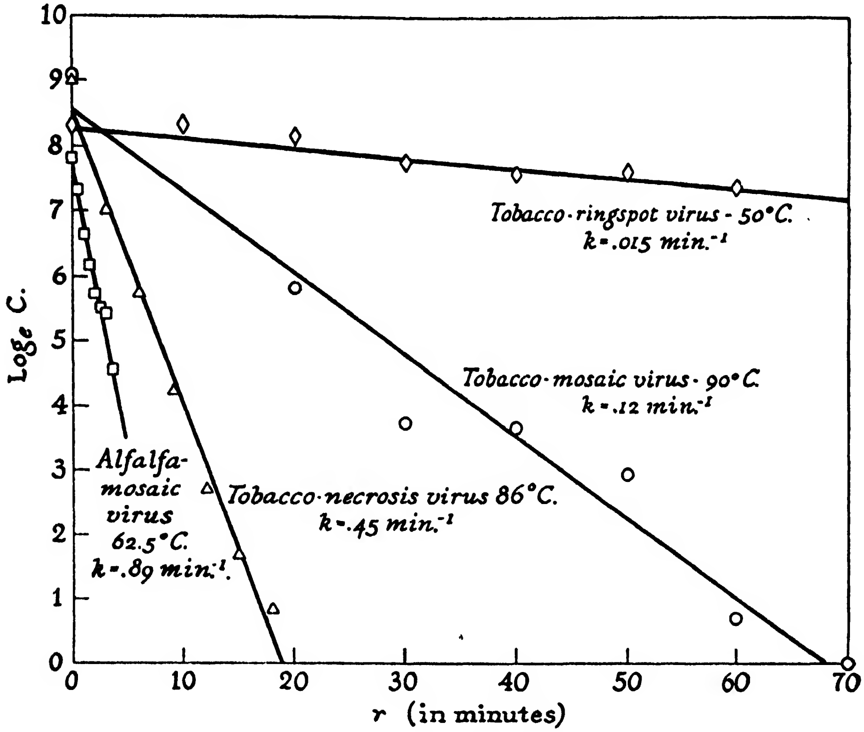

FIG. 1. Curves illustrating the thermal inactivation rate of tobacco-ringspot virus at 50°C., of tobacco-mosaic virus at 90°C., of tobacco-necrosis virus at 86°C., and of alfalfa-mosaic virus at 62.5°C.; all in freshly expressed juice of diseased plants.

the dilution curve is less than unity and that it varies with conditions under which the data are obtained. In the present work, this variation was probably at random in the various tests at each temperature and may account for some of the variation in the calculated inactivation rates. Such random variation would not be expected to affect markedly the accuracy of the mean value of k. The true value of k is the quotient of the calculated value of k divided by the slope of the dilution curve. If the calculated value of k is a simple multiple of the true value, it is just as accurate for comparative purposes.

Comparison of inactivation rates of tobacco-mosaic virus in undiluted plant juice and in juice diluted 1:20 shows that virus concentration markedly affects the rate of inactivation (table 5). The rate of denaturation* of the virus has likewise been found to be affected by virus concentration.[7] The lesion counts of unheated virus samples used in the present study indicate that there was considerable variation in concentration of the samples used in the various tests. This variation probably accounts for some of the differences in estimated k values.

It has been shown that the hydrogen-ion concentration of a tobacco-mosaic virus solution has an effect on rate of denaturation of the virus at a given temperature.[7] The difference found in the thermal inactivation point

TABLE 6

Velocity Constants of Inactivation ($k_{\text{min.}^{-1}}$) for Thermal Inactivation of Tobacco-Mosaic Virus in Dried Air-Cured White Burley Tobacco

Temperature degrees C.	k min.$^{-1}$
150	$3,6 \times 10^{-1}$
140	$2,9 \times 10^{-1}$
130	$2,2 \times 10^{-1}$
120	$1,2 \times 10^{-1}$
110	$2,6 \times 10^{-2}$
100	$9,7 \times 10^{-3}$
90	$7,2 \times 10^{-3}$
80	$1,9 \times 10^{-3}$
70	$9,8 \times 10^{-4}$

of this virus in crude plant juice[10] and in purified preparations[16] is attributed largely to the difference in p_H of these solutions.[7] In the present study, variation in k was not correlated with differences in p_H. The effect of p_H, if any, may have been obscured by the action of other factors.

The temperature coefficient. The temperature coefficient of inactivation is the ratio between velocity constants at two different temperatures—

$$Q_{t0} = \frac{k_2}{k_1} \qquad \text{(Eq. 4)}$$

where Q_{t0} is the temperature coefficient at 2 temperatures differing by t^0C., and k_2 and k_1 are the velocity constants at the higher and lower tempera-

* It has been shown recently[7] that thermal inactivation of tobacco-mosaic virus is not identical with thermal denaturation of this virus. Thermal inactivation is probably one of the earlier reactions in the series which finally leads to denaturation.

tures, respectively. It is customary to calculate the temperature coefficient for an interval of 10°C. Values for Q_{10° may be calculated from any 2 values of k by means of the following equation:

$$\log Q_{10^\circ} = \frac{10(\log k_2 - \log k_1)}{t_2 - t_1} \qquad \text{(Eq. 5)}$$

where t_2 is the higher temperature and t_1 is the lower. Q_{10° generally varies with the temperature range. It may readily be demonstrated with the data presented above that the Q_{10° values for the various viruses, with the possible exception of alfalfa-mosaic virus, are not constant over any appreciable temperature range. A more nearly constant relationship between the rates of chemical reactions and temperature is given by the equation of Arrhenius which may be expressed in the following form:

$$\log_e \frac{k_1}{k_2} = \frac{E}{R}\left(\frac{1}{T_2} - \frac{1}{T_1}\right) \qquad \text{(Eq. 6)}$$

where T is the absolute temperature, R is the gas constant, and E is a constant usually referred to as the energy of activation. According to this equation, when $\log_e k$ is plotted against $\frac{1}{T}$ a straight line should be obtained. This relationship holds generally for chemical reactions. In figure 2, the data for all 4 viruses are plotted in this manner. Straight lines have been fitted to the data for alfalfa-mosaic and tobacco-necrosis viruses. In the cases of the data for tobacco-ringspot and tobacco-mosaic viruses, the best fit is obtained by 2 straight lines of different slope.

The energy of inactivation may be calculated from equation 6 or directly from the curves of figure 2. When this is done, the values given in table 7 are obtained. The high values of E are of the same order as those obtained for denaturation of proteins, inactivation of enzymes, and killing of microorganisms. They are comparable to the value, 101 000 calories per mole, found by *Krueger*[6] for heat inactivation of antistaphylococcus bacteriophage.

The significance of E as a characteristic of the 4 plant viruses. Just as thermal inactivation point is used to characterize a plant virus so may the velocity constant of inactivation at a specified temperature be used for the same purpose. The velocity constant may be determined with greater accuracy than the thermal inactivation point from an equivalent amount of data. A true picture of the heat stability of a plant virus cannot, however, be obtained unless the velocity constants of inactivation of the virus at several different temperatures under specified conditions are known. For

example, reference to figure 2 shows that tobacco-mosaic virus and tobacco-necrosis virus in undiluted plant juice have the same velocity constant of inactivation at a temperature of about 92,7°C. but different constants at other temperatures. The k values for these two viruses change differently

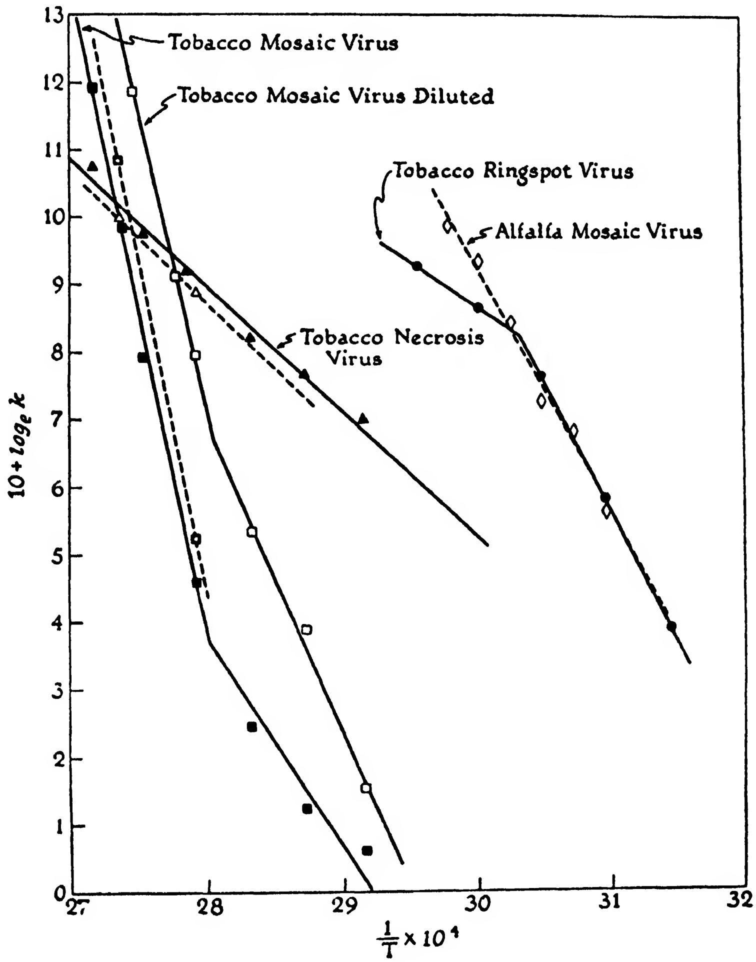

FIG. 2. Curves obtained when the natural logarithms of the inactivation velocity constants of four different plant viruses are plotted against absolute temperature according to the Arrhenius equation.

with temperature. The E value, or energy of activation of the virus, is a measure of the rate of change. The 4 plant viruses studied differ in the magnitude of their E values. Unfortunately, it cannot be determined from the present work whether or not E is constant over a wide temperature

range. Tobacco-mosaic virus shows a sharp change in E at 84°C. for the undiluted material and at 83°C. for the diluted juice. Tobacco-ringspot virus also shows a sharp change in E at 56,5°C., although in the opposite direction. It is possible that the break at these temperatures is not a property of the virus but due to errors in the experimental method or to changes in juice samples held at high temperatures for long periods of time. No such break was found in the E value when thermal denaturation instead of thermal inactivation of tobacco-mosaic virus was studied over a similar temperature range[7].

The E values calculated for tobacco-mosaic and tobacco-necrosis viruses over the temperature range from 84° to 95°C. were 195000 and 37300 calories per mole, respectively (table 7). The E values calculated from inactivation data on a mixture of equal volumes of juice from tobacco plants

TABLE 7

Calculated Energy of Activation Values (E) at Specified Temperature Ranges for 4 Plan Viruses

Virus	Temperature range (degrees C.)	E (calories per mole)
Tobacco necrosis	70–95	37 300
Alfalfa mosaic	50–62,5	75 000
Tobacco ringspot	56,5–65	27 600
	45–56,5	78 800
Tobacco mosaic (undiluted)	84–95	195 000
	68–84	55 300
Tobacco mosaic (diluted)	83–91	176 000
	68–83	92 000
Tobacco mosaic (in dried leaf material)	70–150	24 100

infected with tobacco-mosaic virus and juice from plants infected with tobacco-necrosis virus (table 4) were 200000 calories per mole for tobacco-mosaic virus and 38 800 calories per mole for tobacco-necrosis virus. Thus, the difference in E value between these two viruses is not due to differences in the juices of tobacco plants affected by the viruses. The marked difference in energy of activation values for tobacco-mosaic and tobacco-necrosis viruses is of interest in view of the fact that these 2 viruses have about the same thermal inactivation point but react to aging *in vitro* at room temperature quite differently from each other.

Dilution of tobacco-mosaic virus solutions did not cause an appreciable change in energy of activation although it did result in considerable change in velocity constants of inactivation at specific temperatures (table 5). This may be seen by comparing the curves of figure 2 and by reference to

table 7. At the higher temperatures the difference in E is of the order of 10 per cent; at the lower temperatures it is of the order of 40 per cent.

The E value calculated for thermal inactivation of tobacco-mosaic virus in air-cured White Burley tobacco is 24 120 calories per mole (tables 6 and 7). This value is considerably below that for inactivation of the virus in solution. No attempt is here made to account for this marked difference. *Thornberry*, *Valleau* and *Johnson*[17] suggested that inactivation of virus in dried leaf material may involve a mechanism different from that involved in inactivation of the virus in solution. They also indicate that thermal inactivation of tobacco-mosaic virus is more rapid when the virus is in crude juice than when in dried leaves. Comparison of the velocity constants of tables 5 and 6 shows that while this is true at high temperatures, just the reverse is true at temperatures below about 80°C. Virus in dried leaf material is inactivated about 1,7 times as rapidly as virus in expressed plant juice at 80°C. and more than 8 times as rapidly at 70°C.

The E value calculated for alfalfa-mosaic virus is intermediate between the values for tobacco-mosaic and tobacco-necrosis viruses. It is essentially equivalent to the E value calculated for tobacco-ringspot virus over the temperature range 45–56,5°C. but nearly 3 times the value of E estimated for tobacco-ringspot virus over the range 56,5–65°C. The E value for tobacco-ringspot virus at the higher temperatures is less than that for tobacco-necrosis virus.

DISCUSSION

The significance of the findings reported in this paper may become clearer by consideration of present theories regarding heat denaturation. It has been shown that thermal inactivation of the viruses studied follows the course of a first order reaction: that is, the rate of inactivation at any given instant is proportional to the concentration of virus at that instant. According to present theories[1], the virus particles in a sample of juice from diseased plants may be thought of as having different energy levels. The distribution of the different levels of energy is in accordance with the laws of probability and may be shown graphically with *Maxwell*'s distribution curve. When energy is supplied to a virus solution by heating, there is a shift in the distribution curve. The proportion of particles with energy levels above E, the energy of activation, is increased. Only those particles that have energy levels greater than E can become inactivated. The more the temperature is increased, the greater is the shift in the energy distribution curve and the greater is the proportional increase in particles having high energy levels. Thus, the rate of inactivation becomes faster as the

temperature is raised. According to the theory outlined above, E should be a constant. The fact that it was not found to be constant in all cases in the present study may be of significance or it may be due to experimental error. Further work is needed to settle this question.

This picture of virus inactivation may be complicated by the fact that viruses are known to produce variants[4]. If the variants in a virus solution require different energy levels for inactivation, the course of the reaction will be changed depending upon the number of such variants in the solution. However, the number of variant particles in a virus solution is thought to be small in relation to the total number of virus particles.

The thermal inactivation point of a plant virus is usually defined as the temperature required to inactivate the virus after an exposure of 10 minutes. Obviously the temperature required will be different if any other time is specified. The thermal inactivation point of a virus depends not only upon the thermo-stability of the virus but also upon the minimum concentration of virus that can be detected with the methods used. The thermal inactivation point thus represents the particular temperature at which the velocity constant is such that the virus content will, in a period of 10 minutes, be lowered beyond the minimum that can be detected. If this minimum content is known, the thermal inactivation point may be predicted from curves such as those shown in figure 2.

The present work does not allow an accurate estimation to be made of the influence of environmental conditions on the E value of the viruses studied. Nevertheless, the E value may prove to be a useful distinguishing characteristic of viruses.

SUMMARY

Thermal inactivations of tobacco-necrosis virus, tobacco-mosaic virus, alfalfa-mosaic virus and tobacco-ringspot virus were found to follow the course of a first order reaction. The rates of inactivation at a number of different temperatures were calculated for each of the 4 viruses. The energy of activation, or E value, for each was calculated from inactivation rates at different temperatures. The E values, in calories per mole, were as follows: for tobacco-necrosis virus at 70–95°C., 37 300; for tobacco-mosaic virus in undiluted juice at 68–84°C. and 84–95°C., 55 300 and 195 000, respectively; in juice diluted 1:20 at 68–83°C. and 83–91°C., 92 000 and 176 000, respectively; in dried leaf material at 70–150°C., 24 100; for alfalfa-mosaic virus at 50–62,5°C., 75 000; and for tobacco-ringspot virus at 45–56,5°C. and 56,5–65°C., 78 800 and 27 600, respectively.

BIBLIOGRAPHY

[1] *Getman, F. H.*, and *F. Daniels:* Outlines of theoretical chemistry. 643 pp. John Wiley and Sons, Inc., New York, 5th Ed., 1931.—[2] *Henderson, R. G.* and *S. A. Wingard:* Further studies on tobacco ringspot in Virginia. J. agr. Res. **43**, 191 (1931).—[3] *Holmes, F. O.:* Handbook of Phytopathogenic Viruses. 221 pp. Burgess Publishing Co., Minneapolis, Minn. 1939.—[4] *Jensen, J. H.:* Isolation of yellow-mosaic viruses from plants infected with tobacco mosaic. Phytopathology **23**, 964 (1933).—[5] *Johnson, James* and *Theodore J. Grant:* The properties of plant viruses from different host species. Phytopathology **22**, 741 (1932).—[6] *Krueger, A. P.:* The heat inactivation of antistaphylococcus bacteriophage. J. gen. Physiol. (Am.) **15**, 363 (1931–32).—[7] *Lauffer, M. A.* and *W. C. Price:* The thermal denaturation of tobacco mosaic virus. *J. Biol. Chem.* (Am.) **133**, 1 (1940). [8] *Loring, H. S.:* Accuracy in the measurement of the activity of tobacco mosaic virus protein. J. biol. Chem. (Am.) **121**, 637 (1939).—[9] *Pierce, W. H.:* Viroses of the bean. Phytopathology **24**, 87 (1933).—[10] *Price, W. C.:* The thermal death rate of tobacco-mosaic virus. Phytopathology **23**, 749 (1933).—[11] *Price, W. C.:* Studies on the virus of tobacco necrosis. Amer. J. Bot. **25**, 603 (1938).—[12] *Price, W. C.:* Comparison of thermal inactivation rates of two plant viruses. Phytopathology **29**, 20 (1939).—[13] *Samuel, G.* and *J. G. Bald:* On the use of the primary lesions in quantitative work with two plant viruses. Ann. appl. Biol. **20**, 70 (1933).—[14] *Smith, K. M.* and *J. G. Bald:* A description of a necrotic virus disease affecting tobacco and other plants. Parasitology **27**, 231 (1935).—[15] *Smith, K. M.:* A textbook of plant viruses. P. Blakiston's Son & Co., Inc., Philadelphia. 1937.—[16] *Stanley, W. M.* and *H. S. Loring:* Properties of virus proteins. Cold Spring Harbor Symp. Quant. Biol. **6**, 341 (1938).—[17] *Thornberry, H. H., W. D. Valleau* and *E. M. Johnson:* Inactivation of tobacco-mosaic virus in cured tobacco leaves by dry heat. Phytopathology **28**, 129 (1938).—[18] *Zaumeyer, W. J.* and *B. L. Wade:* The relationship of certain legume mosaics to bean. J. agr. Res. **51**, 715 (1935).

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THE RELATIONSHIP BETWEEN VIRUSES OF POTATO CALICO AND ALFALFA MOSAIC

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It was observed that potato-calico virus produces symptoms in *Nicotiana glutinosa* L. very similar to those caused by alfalfa-mosaic virus (*Marmor medicaginis* H.)[1] in the same host plant. This observation suggested that the two viruses might be closely related. It is the purpose of this paper to point out the similarities and differences in the symptoms of the two diseases and to report the results of cross protection tests. The investigation showed that the two viruses, although distinct, are indeed closely related.

Virus Stocks

Potato-calico virus was obtained from E. S. Schultz. It is apparently identical with that studied by Porter (5, 6, 7) and Dykstra (2). On the basis of symptomatology, Porter (6) distinguished between the viruses of potato-calico and potato-aucuba mosaic (*Marmor aucuba* H.). Dykstra (2) likewise distinguished between these viruses and reported further that potato-calico virus is apparently unrelated to the viruses of potato-aucuba mosaic and Canada-streak (*M. aucuba* H. var. *canadense*, n. var.),[2] a conclusion which is confirmed in the present paper.

Alfalfa-mosaic virus was secured from H. T. Osborn. It is believed to be the same as or a closely related strain of that studied by Zaumeyer and Wade (10) and Zaumeyer (11) and designated by the latter as alfalfa-mosaic virus 1. The literature is somewhat confused as to whether there are one or two alfalfa-mosaic viruses. Pierce (4) distinguished between the alfalfa-mosaic virus studied by Weimer (8, 9) and that studied by himself

[1] Latin binomials used in this paper are based on the system of nomenclature in the Handbook of Phytopathogenic Viruses (3).

[2] It has been shown (1, 2) that the viruses of potato-aucuba mosaic and Canada-streak are related. The comparative symptomatology of the induced diseases clearly indicates that the viruses are not identical. For this reason, it is felt that the Canada-streak strain of virus should have varietal rank. The name *canadense*, suggested by the common name, seems appropriate.

and accordingly referred to the viruses as alfalfa viruses 1 and 2, respectively. Zaumeyer (11), on the other hand, considered Weimer's virus and Pierce's virus to be the same and referred both to alfalfa-mosaic virus 1. The writers agree that there is insufficient evidence for distinguishing between alfalfa viruses 1 and 2.

Comparative Symptomatology

Both potato-calico virus and alfalfa-mosaic virus were transmitted to several species of plants by the rubbing method of inoculation. In most cases carborundum was employed. Both viruses were readily transferred from young *Nicotiana glutinosa* plants to other plants of the same species. They produced almost identical symptoms after an incubation period of 3 to 4 days. *N. glutinosa* proved to be a useful test plant and a good source plant for both viruses. On beans (*Phaseolus vulgaris* L. var. Early Golden Cluster and Corbett Refugee) and on Black Eye cowpea (*Vigna sinensis* Endl.) they produced the same type of necrotic primary lesion. On beans, lesions sometimes appeared within 24 hours after inoculation. Both viruses produced necrotic primary lesions followed by a systemic streak disease in broad bean (*Vicia faba* L.). On seedlings of Green Mountain potatoes (*Solanum tuberosum* L.), they produced similar symptoms but the symptoms of potato-calico virus were more severe than those caused by alfalfa-mosaic virus. Both viruses caused mottling and necrotic vein-banding in crimson clover (*Trifolium incarnatum* L.) and in red clover (*T. pratense* L.). Both produced a mottling disease in white clover (*T. repens* L.), and bright yellow spotting in leaves of the Improved Long Green cucumber (*Cucumis sativus* L.). The similarity in the rather distinctive reactions of the 2 viruses in these hosts strongly suggested that they might represent strains of one virus.

That potato-calico and alfalfa-mosaic viruses are not identical is shown by the fact that the former is the milder of the two in *Nicotiana glutinosa*, crimson clover, and red clover, whereas the latter is the milder in potato. Moreover, under comparable conditions, the potato-calico virus produces fewer lesions in kidney beans, broad beans and cowpeas than does alfalfa-mosaic virus.

Cross Protection Tests

Cross-inoculation experiments were made on *Nicotiana glutinosa* and *N. tabacum* L. var. Turkish. It should be pointed out that in *N. glutinosa* both viruses cause diseases showing an acute stage with severe symptoms

followed by a chronic stage with mild symptoms. Groups of six young *N. glutinosa* plants were inoculated with one or another of the viruses causing the following diseases: Alfalfa-mosaic, potato-calico, potato-ringspot (caused by ***Marmor dubium*** H. var. ***annulus*** H.), cucumber-mosaic (caused by ***M. cucumeris*** H. var. ***vulgare*** H.), and Canada-streak. Juice

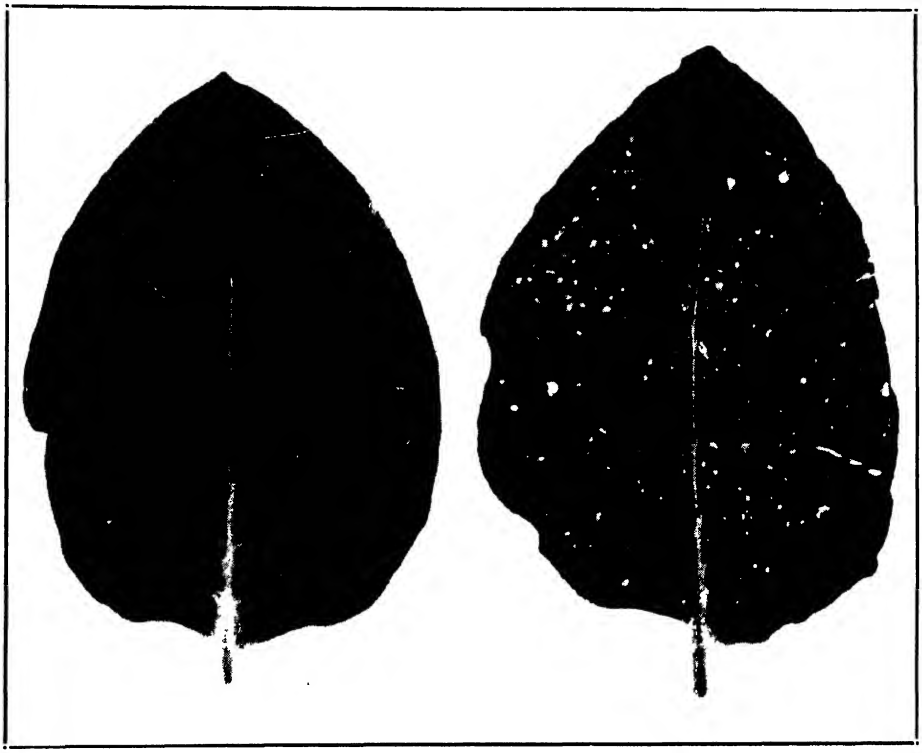

FIG. 1. Leaves from a cross inoculation test on *Nicotiana tabacum* var. Turkish. Both leaves were inoculated with alfalfa-mosaic virus. The leaf on the right had previously been healthy, that on the left had been invaded by the virus of potato-calico. The symptoms of potato-calico were mild and do not show in the photograph. (Photograph by J. A. Carlile.)

from healthy *N. glutinosa* plants was rubbed over the leaves of six additional plants. Twelve days later, when the inoculated plants were systemically infected, three upper leaves on each of three plants in each group were inoculated with juice from *N. glutinosa* plants infected with alfalfa-mosaic virus. Leaves on the remaining three control plants in each group were similarly rubbed with juice from healthy *N. glutinosa* plants. The plants were observed for two weeks. None of the control plants rubbed with

juice from healthy plants developed additional symptoms. Of the leaves rubbed with juice containing alfalfa-mosaic virus, those previously infected by potato-calico virus or alfalfa-mosaic virus were alive and green at the end of this period; the others were dead or moribund. Moreover, the new leaves on the plants previously infected with potato-calico or alfalfa-mosaic showed no symptoms in addition to those characteristic of the chronic stages of these diseases while new leaves on the other plants developed systemic necrotic lesions typical of the acute stage of alfalfa-mosaic.

Similar results were obtained when plants of *Nicotiana tabacum* var. Turkish that had previously been infected with the potato-calico virus were inoculated with alfalfa-mosaic virus. The inoculated leaves of these plants developed no necrotic primary lesions, whereas leaves of previously healthy plants (Fig. 1) or plants infected with cucumber-mosaic virus developed many such lesions.

The protection described above is considered good evidence that potato-calico and alfalfa-mosaic viruses are closely related. The potato-calico virus is, therefore, classified as a strain of *Marmor medicaginis* and given the varietal name *solani* from NL. *Solanum*, generic name for the potato. Alfalfa-mosaic virus should be designated as *M. medicaginis* H. var. *typicum* n. var. to distinguish it from the potato-calico strain of the virus.

SUMMARY

Potato-calico virus and alfalfa-mosaic virus produce similar but not identical symptoms on *Nicotiana glutinosa* L., *Phaseolus vulgaris* L., *Vicia faba* L., *Vigna sinensis* Endl., *Solanum tuberosum* L., *Trifolium incarnatum* L., *T. pratense* L., *T. repens* L., and *Cucumis sativus* L.

Plants of *Nicotiana glutinosa* and *N. tabacum* infected with potato-calico virus are refractory to infection with alfalfa-mosaic virus. Potato-calico and alfalfa-mosaic viruses are, therefore, considered to be closely related and the potato-calico strain is named *Marmor medicaginis* H. var. *solani* n. var. Plants affected by potato-ringspot, cucumber-mosaic or Canada-streak are susceptible to infection with alfalfa-mosaic virus. Therefore, the viruses causing these diseases are not thought to be closely related to alfalfa-mosaic virus.

LITERATURE CITED

1. Chester, K. S. Serological studies of plant viruses. Phytopath. **27,** 903–912. 1937.
2. Dykstra, T. P. A study of viruses causing yellow mosaics in European and American varieties of the potato, *Solanum tuberosum*. Phytopath. **29,** 917–933. 1939.

3. Holmes, F. O. Handbook of Phytopathogenic Viruses. 221 p. Burgess Publishing Co. (Minneapolis). 1939.
4. Pierce, W. H. Viroses of the bean. Phytopath. **24,** 87-115. 1934.
5. Porter, D. R. Recent investigations of potato calico. Potato Assoc. Amer. Proc. **18,** 65-69. 1931.
6. ———. The infectious nature of potato calico. Hilgardia **6,** 277-294. 1931.
7. ———. Insect transmission, host range, and field spread of potato calico. Hilgardia **9,** 383-394. 1935.
8. Weimer, J. L. Alfalfa mosaic. Phytopath. **21,** 122-123. 1931.
9. ———. Studies on alfalfa mosaic. Phytopath. **24,** 239-247. 1934.
10. Zaumeyer, W. J., and Wade, B. L. The relationship of certain legume mosaics to bean. Jour. Agr. Res. [U. S.] **51,** 715-749. 1935.
11. ———. A streak disease of peas and its relation to several strains of alfalfa mosaic virus. Jour. Agr. Res. [U. S.] **56,** 747-772. 1938.

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STRAINS OF POTATO YELLOW-DWARF VIRUS

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THE WORK of Carsner (1925) on attenuation of the curly-top virus (*Chlorogenus eutetticola* H.),[1] of Storey and McClean (1930) on strains of maize-streak virus (*Marmor maidis* H.), and of McKinney (1926) and Jensen (1933) on strains of tobacco-mosaic virus (*M. tabaci* H.) has been followed by a number of papers showing that plant viruses commonly give rise to variants. Recent studies (Black, 1937 and 1938) on the potato yellow-dwarf disease led to the isolation of 7 distinct strains of the causal virus (*M. vastans* H.). The purpose of this paper is to discuss the origin of these strains, to describe their symptoms, and to suggest their utility in cross-inoculation studies with viruses suspected of being related to that of potato yellow dwarf.

ORIGIN OF THE STRAINS.—The strains dealt with in this paper represent only those isolants which are considered distinct. Isolants which did not appear to be distinctive were discarded. Potato yellow-dwarf virus strains B_1 and B_2 were obtained from a *Nicotiana glutinosa* L. plant inoculated by means of the clover leafhopper (*Aceratagallia sanguinolenta* Prov.). The plant showed unusually mild symptoms. Selected mildly-affected scions were used to inoculate other *N. glutinosa* plants. After several serial passages in plants inoculated by grafting had demonstrated that two virus strains distinctly milder than the field strain had been obtained, the strains were transferred to and maintained in *N. rustica* L. plants. Inoculations on *N. rustica* were made by the rubbing method using carborundum as an abrasive. Two additional strains, B_3 and B_7, were obtained by passing the field strain through Turkish tobacco plants (*N. tabacum* L.) and chicory plants (*Cichorium intybus* L. var. Large Rooted), respectively. Two strains, B_4 and B_5, were obtained by isolation from local lesions produced in *N. rustica* leaves inoculated with the field strain of the virus. A leaf bearing several hundred typical diffuse yellow lesions resulting from inoculation with the field strain frequently shows one or more variant lesions.

[1] In this paper viruses are named according to the system of nomenclature developed by Holmes (1939).

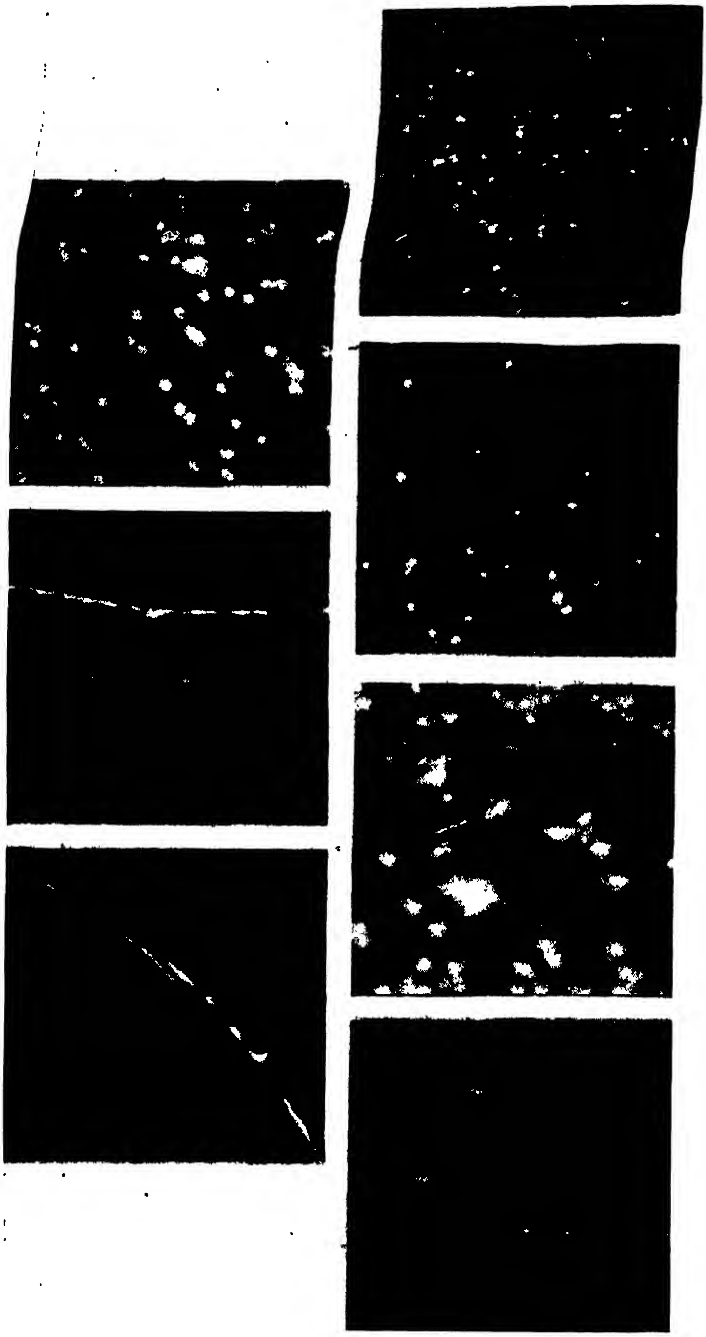

Fig. 1. Local lesions of different strains of potato yellow-dwarf virus in *Nicotiana rustica*. From left to right, the strains represented are: top row, B_1, B_2, B_3; bottom row, B_4, B_5, B_6, and B_7. Photographs by J. A. Carlile.

From such leaves some typical yellow lesions and some necrotic brown lesions were excised by means of sterile cork borers. The two groups of lesions were employed separately as inoculum. In each case the local lesions developing in the inoculated plants showed a high proportion of lesions of the type used as a source of inoculum. This process was repeated until plants were obtained exhibiting only lesions of one type or the other. The strain producing the yellow lesion was called B_4. This strain is similar to, if not identical with, the virus as it occurs in the field. It continued to produce occasional brown necrotic lesions. The strain producing only brown necrotic lesions was designated B_5. Strain B_6 was obtained from a single clover leafhopper which transmitted a necrotic type variant of potato yellow-dwarf virus to crimson clover plants (*Trifolium incarnatum* L.).

Since the potato yellow-dwarf virus is not confined to the primary lesions, virus from many local lesions undoubtedly becomes mixed in the systemically invaded portions of the plant. Consequently a strain probably has the greatest chance of being pure when it develops in a plant bearing a single local lesion. After the 7 strains had been established in *Nicotiana rustica*, those showing variability in their local lesions (strains B_1 to B_5) were purified by starting them from a plant bearing a single primary lesion, as was the case with strains B_3 and B_5, or from a plant bearing 2 or 3 apparently identical lesions, as was the case with strains B_1, B_2, and B_4. From the beginning, strains B_6 and B_7 showed remarkably uniform local lesions and no attempt was made to purify them in this way.

Description of symptoms on Nicotiana rustica. The symptoms caused by a single strain of the potato yellow-dwarf virus in *Nicotiana rustica* varied somewhat from plant to plant and were affected to some extent by nutrition and season. The plants infected by the different strains were all grown in composted soil in 6-inch pots kept in a greenhouse held at about 27°C. Most of the time the temperature was within 3°C. of this figure. Early in the study an experiment was carried out in which each of the strains B_1 to B_6 was inoculated into 5 *N. rustica* plants and the symptoms induced by them compared under identical conditions. Observations were confirmed in a second similar experiment. Strain B_7 was isolated after these experiments were carried out. However, it was subsequently maintained on *N. rustica* in the greenhouse along with strains B_1 to B_5 for about 18 months. Strain B_6 was lost during this period. Meanwhile, to maintain the strains, each was transferred about every two months to 3 healthy *N. rustica* plants. There was ample opportunity in this interval to observe that the seven strains were continuously and constantly different,

TABLE 1

Symptoms of Potato Yellow-Dwarf Virus Strains on Nicotiana rustica L. and on Solanum tuberosum L.

Strain	Symptoms on *Nicotiana rustica* L.: Local lesions	Symptoms on *Nicotiana rustica* L.: Systemic symptoms	Symptoms on *Solanum tuberosum* L. var. Green Mountain
B_1	Light green, diffuse.	Mild. Leaves turned yellow slowly.	Slight vein clearing, slight necrosis of apical pith. Leaves turned yellow with little vein necrosis. Top of plant eventually died. Axillary shoots developed with chronic stage of disease. Plants produced viable tubers. Progress of disease slow.
B_2	Like those of B_1 but milder.	Like those of B_1 but milder.	Like those of strain B_1 but the disease progressed more rapidly and the transition to the chronic stage in the axillary shoots did not occur so readily. Plants produced no viable tubers.
B_3	Small, yellow, sometimes with necrotic margins.	Leaves yellow only at tip; rest of leaf green except for vein clearing and scattered yellow or brown spots.	Like those of strain B_1. Virus not so readily transmitted to potato as strain B_1.
B_4	Large, diffuse, yellow.	Leaves turned yellow fairly quickly.	Like those of strain B_1 but somewhat more severe.
B_5	Brown, necrotic.	Extensive vein necrosis quickly followed by yellowing and death of the leaf.	Black necrosis of veins quickly followed by yellowing and collapse of the whole leaf. Severe necrosis of apical pith. Top of plant killed quickly. Many axillary buds killed instead of developing in the chronic stage. No viable tubers produced. Progress of disease rapid.
B_6	Brown, necrotic, much smaller than those of strain B_5.	Systemic symptoms delayed. Vein enlargement and necrosis followed by yellowing and death of leaves. More severe than strain B_5.	Diseased *N. rustica* scions failed to unite with potato and no infections were obtained.
B_7	Small, light yellow, with irregular margins.	Systemic symptoms delayed. Intense yellowing of leaves.	No attempt was made to transmit strain to potato.

each from the other. Photographs of the symptoms produced in *N. rustica* plants were made in the spring of the year between March 1 and May 16. With the exception of the local lesions of strain B_7, the lesions shown in figure 1 resulted from inoculations made on the same day and photographed 16 days later. The lesions of strain B_7 shown in figure 1 were photographed during the same season a year later.

The distinctive local lesion symptoms (fig. 1) and systemic symptoms of the potato yellow-dwarf virus strains in *Nicotiana rustica* are summarized in table 1 and described briefly as follows:

Strain B_1 produced a light green diffuse local lesion. Systemic symptoms were mild and the yellowing of invaded leaves proceeded slowly (fig. 2a).

Strain B_2 is very similar to strain B_1, but somewhat milder. The local lesions were sometimes difficult to detect, and the systemic symptoms were distinctly milder than those of strain B_1.

Strain B_3 produced a small yellowish lesion with a definite outline. Under some conditions the lesion was characterized by a partly necrotic ring near the border and by a yellow or green center. A leaf systemically invaded by this strain usually showed yellowing at the tip, the rest of the leaf showing clearing of the veins and yellow or brownish spots.

Strain B_4 caused large diffuse yellow lesions. Systemically invaded leaves turned uniformly yellow rather quickly.

Strain B_5 produced brown lesions which soon developed necrotic gray centers. This strain caused a rapid yellowing of the leaf, extensive vein necrosis, the formation of large necrotic areas, and finally death of the whole leaf (fig. 2b). Sometimes the entire plant was killed.

Strain B_6 was more difficult to transfer than the others and eventually was lost. It produced necrotic lesions much smaller than those of strain B_5. The appearance of systemic symptoms was delayed. Apparently this virus was slower in invading the plant than was the field strain. Systemic symptoms were more severe than those of strain B_5. Vein necrosis was extensive and rapid and apparently was preceded by vein enlargement. The dead veins formed a raised netting on the leaves. This condition was rapidly followed by large necrotic blotches and death of the whole leaf. If systemic invasion occurred before the plant matured, the plant was killed.

Strain B_7 formed small light yellow lesions with margins that branched unevenly. Systemic symptoms consisted of a pronounced yellowing of the invaded leaves. When first isolated this strain was very difficult to transfer and unsuccessful inoculations were common. The virus invaded the plant more slowly than strain B_6 and usually it was necessary to force new growth several times by pruning before systemic symptoms would appear. With

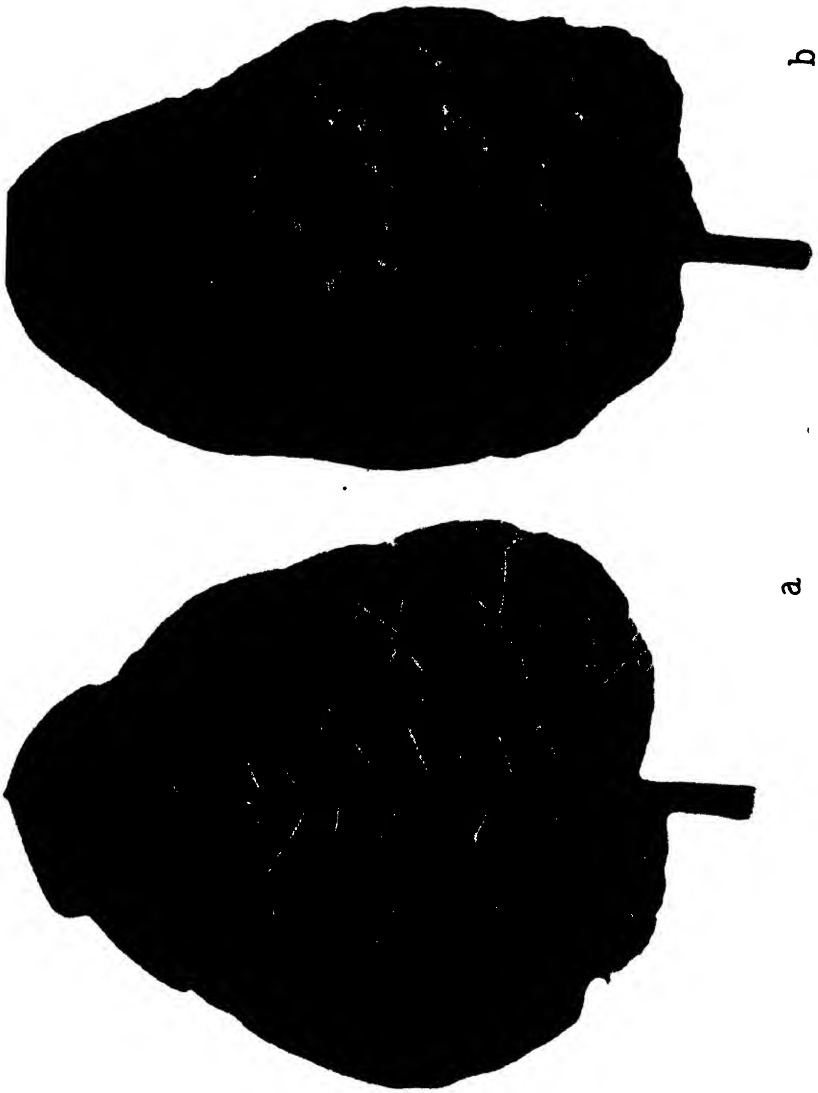

Fig. 2. (a) Symptoms of strain B_1 in a leaf of *Nicotiana rustica* 6 days after systemic invasion was first detected. (b) Symptoms of strain B_5 in a leaf of *N. rustica* 6 days after systemic invasion was first detected. Photographs

repeated passage in *Nicotiana rustica*, the strain, or more probably a derivative of it, became more easily transmissible and more quickly invasive.

Almost any *Nicotiana rustica* leaf bearing numerous lesions as a result of inoculation with the field strain of yellow-dwarf virus shows a few lesions of atypical appearance. The isolation of different strains of the virus with distinctive local lesions, as described above, indicates that the atypical lesions resulting from inoculation with the field strain (or some other strain) are due to mutants. Virus from a few isolated mutant lesions possibly rarely invades the host plant systemically because of the much greater chance that the parent strain, occurring in a great many local lesions, will invade systemically first and thus protect against invasion by the variant. The persistent occurrence of variants in inoculum from systemically invaded leaves suggests that the mutants arise frequently from the parent strain in the systemically invaded portions of the plant. The appearance of the strain B_5 type of lesion on many leaves inoculated with the field strain suggests that this mutant occurs frequently when the virus is developing in *N. rustica*.

Description of symptoms on Solanum tuberosum.—Small pieces of the stems of *Nicotiana rustica* plants infected with strains B_1 to B_6 were used as scions in grafting experiments on potato plants (*Solanum tuberosum* L. var. Green Mountain). In this way 17 potato plants were inoculated with each of these strains and, with the exception of strain B_6, each strain was successfully carried to 10 or more Green Mountain plants. The inoculations with strain B_6 failed entirely, probably because this necrotic strain was so severe on *N. rustica* that the scions died before they united with the stocks. Strain B_7 was discovered after these experiments were carried out, and no attempt was made to transfer it to potato.

Each of the 5 strains transmitted to *Solanum tuberosum* produced a severe disease. The symptoms are summarized in table 1. Although there was considerable variation within each strain, the milder strains (B_1, B_2, B_3, and B_4) tended to have a longer incubation period, milder symptoms, and a much slower progression of symptoms than the severe strain B_5. The milder strains produced less necrosis in the leaf veins and in the pith of the growing tip than did strain B_5. Leaves infected with the mild strain B_2 remained alive for a week or more after the appearance of the first symptoms, whereas leaves infected with the severe strain B_5 often collapsed and died a day or two after the first appearance of vein necrosis (fig. 3). The axillary branches of plants infected with strains B_1, B_2, B_3, and B_4 usually grew and showed symptoms of the chronic stage. In plants infected with strain B_5, on the other hand, the axillary buds were generally killed.

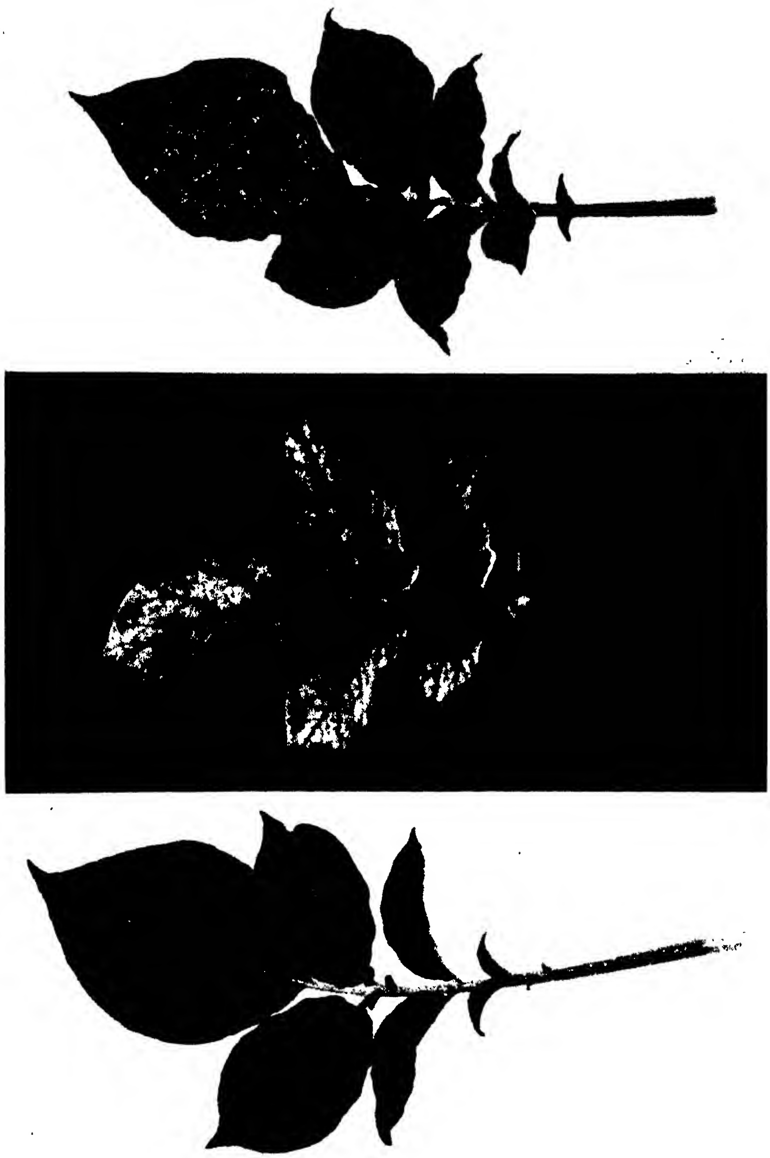

FIG. 3. Symptoms of potato yellow-dwarf virus strains in *Solanum tuberosum* var. Green Mountain. From left to right: a healthy leaf, a leaf infected with strain B_2, and a leaf infected with strain B_5. Photographs by J. A. Carlile.

Plants invaded by strains B_2 and B_6 produced tubers that rotted completely before harvest or during storage. It is surprising that the mild strain B_2 should have had such a destructive action on the tubers. When affected by strains B_1, B_3, and B_4, the plants produced viable tubers which later gave rise to vines exhibiting chronic symptoms resembling those caused by the field strain of the virus. None of the mild strains was sufficiently attenuated to be useful for the purpose of vaccinating potatoes against the field strain of the virus.

CROSS-INOCULATION STUDIES. Two experiments were carried out to ascertain whether or not *Nicotiana rustica* plants invaded by one yellow-dwarf virus strain were specifically protected against the entrance of a second strain. In the first experiment each of the strains B_1, B_2, B_3, and B_4 was inoculated by the rubbing method into 6 young *N. rustica* plants. The leaves of 6 control plants were similarly rubbed with juice from healthy *N. rustica.* The plants were repeatedly pruned and fed a complete nutrient solution to carry them through the acute stage of the disease into the chronic stage while still in a vigorous vegetative period of growth. Three of the plants in each group were held as controls, and 3 were inoculated with the necrotic strain B_6 by rubbing infectious juice on 3 leaves of each plant. A few local lesions of strain B_5 were produced on some of the inoculated leaves previously invaded with the other strains of yellow dwarf virus, indicating that these leaves had been partially but not completely invaded by the first strain. The 3 plants which were healthy previous to being inoculated with strain B_6 developed numerous local lesions on the inoculated leaves and were the only plants in the experiment which subsequently developed systemic symptoms of strain B_5. The plants previously invaded by strains B_1, B_2, B_3, and B_4 were protected against systemic invasion by strain B_5 and could not be differentiated in this respect from the controls uninoculated with strain B_5.

In a second experiment carried out in a similar manner the necrotic strain B_5 was inoculated into groups of 3 *Nicotiana rustica* plants, each previously invaded by one of the following viruses: potato-ringspot (*Marmor dubium* H. var. *annulus* H.), potato-veinbanding (*M. cucumeris* H. var. *upsilon* H.), cucumber-mosaic (*M. cucumeris* H. var. *vulgare* H.), alfalfa-mosaic (*M. medicaginis* H. var. *typicum* Black and Price) (Black and Price, 1940), potato yellow-dwarf strain B_2, potato yellow-dwarf strain B_7; and into one group of 3 healthy plants. Controls consisted of similar groups of 3 plants each which were not inoculated with strain B_5. Only a few local lesions of strain B_5 developed on the inoculated leaves previously invaded by the strains of potato yellow-dwarf virus. Such lesions were numerous

FIG. 4. Protection by a mild strain. The *Nicotiana rustica* plant on the left was invaded by strain B_1 before

on all the other leaves inoculated with strain B_5, although their development was somewhat impeded on leaves previously invaded by potato-veinbanding or cucumber-mosaic viruses. Within one month after inoculation with strain B_5 advanced systemic symptoms of this strain developed on all plants inoculated with it, except the plants previously invaded by the milder strains of potato yellow-dwarf virus. The latter plants were observed for 3 months after inoculation with strain B_5. Figure 4 illustrates the protective action of a mild strain of yellow-dwarf virus.

These experiments show that potato yellow-dwarf virus strains B_1, B_2, B_3, B_4, and B_7 protected *Nicotiana rustica* plants against systemic invasion by strain B_5, whereas several other presumably unrelated viruses did not. They, therefore, seem to provide a means for determining the relationship of potato yellow-dwarf virus to other viruses capable of systemic invasion of this plant.

NOMENCLATURE.—Although each of the 7 described strains of the potato yellow-dwarf virus appeared to be distinct, it seems unwise to give each of them a varietal name. Some may be lost and never recovered again with certainty. Some may have no other utility than that of showing the variability of the parent virus. It seems better at present to name only those strains which offer some prospect of permanence or usefulness. Only two of the potato yellow-dwarf strains described here, B_4 and B_5, seem to merit varietal names at the present time. They can probably always be readily isolated from local lesions in *Nicotiana rustica* leaves inoculated with the field strain of the virus. Their utility in cross-inoculation studies has been demonstrated. Therefore, the field strain B_4 is given the name *Marmor vastans* H. var. *vulgare* n. var. and strain B_5 the name *M. vastans* H. var. *lethale* n. var.

SUMMARY

Seven strains of potato yellow-dwarf virus have been isolated. These differ in respect to the appearance of the local lesions and systemic effects they produce in *Nicotiana rustica*. Some strains cause milder symptoms on Green Mountain potatoes than do others, but all cause a rather severe disease on this plant.

Five strains of the virus protected *Nicotiana rustica* plants from subsequent systemic invasion by a necrosing strain. The presumably unrelated viruses, potato-ringspot, potato-veinbanding, cucumber-mosaic, and alfalfa-mosaic, did not afford protection.

The field strain of the virus is named *Marmor vastans* H. var. *vulgare*. A strain producing necrotic local lesions in leaves of *Nicotiana rustica* is described and named *M. vastans* H. var. *lethale*.

LITERATURE CITED

Black, L. M. 1937. A study of potato yellow dwarf in New York. New York (Cornell) Agric. Exp. Sta. Mem. 209: 1-23.

———. 1938. Properties of the potato yellow-dwarf virus. Phytopathology 28: 863-874.

———, and W. C. Price. 1940. The relationship between viruses of potato calico and alfalfa mosaic. Phytopathology 30: 444-447.

Carsner, E. 1925. Attenuation of the virus of sugar beet curly-top. Phytopathology 15: 745-758.

Holmes, F. O. 1939. Handbook of phytopathogenic viruses. Burgess Publishing Co., Minneapolis. 221 pp.

Jensen, J. H. 1933. Isolation of yellow-mosaic viruses from plants infected with tobacco mosaic. Phytopathology 23: 964-974.

McKinney, H. H. 1926. Virus mixtures that may not be detected in young tobacco plants. Phytopathology 16: 893.

Storey, H. H., and A. P. D. McClean. 1930. The transmission of streak disease between maize, sugar cane and wild grasses. Annals App. Biol. 17: 691-719.

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DOES "C.P. GRADE" SUCROSE CONTAIN IMPURITIES SIGNIFICANT FOR THE NUTRITION OF EXCISED TOMATO ROOTS?

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Ever since the early work of Kotte (2) and Robbins (4) on the limited cultivation of excised roots there has persisted the conviction, formulated at that time, that growth of such roots is dependent on unknown as well as known factors. Robbins (5), Robbins and Maneval (7, 8) and later Robbins and V. B. White (10) concluded that growth of excised corn roots was dependent on some unknown material not contained in the salts of Pfeffer's solution, glucose, water, dissolved gases, peptone, or autolyzed yeast. It has subsequently been shown that continued growth of excised roots of many plants other than corn can be obtained in a nutrient containing these same salts, brewer's yeast, and sucrose (12, 14) and that roots of tomato, sunflower, and pea can be grown in a similar salt solution plus cane sugar, thiamin, and glycine, or other amino acids (17). Robbins and Schmidt (9), and Fiedler (1) nevertheless return to this idea of unknown essentials in discussing recent results obtained with tomato roots and postulate the existence of such unknowns in the carbohydrates used.

This interpretation may, of course, be sound. But it appears not to be based on actual evidence. It should, however, be possible to test this question. If the growth obtained with certain sugars is attributable to the presence of impurities, as suggested, then progressive removal of these impurities should result in progressively poorer and poorer growth. To obtain evidence bearing on this question, a sample of sucrose *purissima* was obtained from the U. S. Bureau of Standards in Washington, D. C.[1] This is their "Standard Sample no. 17" prepared for use in molecular weight determinations, calorimetric studies, etc. Its designated properties are:

specific rotation $[\alpha]\frac{20°C.}{546.1\ m\mu} = 78°.342$ when C = 26 gm. in 100 ml., heat of combustion 3941

$$[\alpha]\frac{20°C.}{589.25\ m\mu} = 66°.529$$

[1] I am indebted to Dr. Eugene Pacsu, Professor of Organic Chemistry in Princeton University and specialist in the chemistry of sugars, for advice and counsel on this subject.

calories per gram weight in air; lot no. 3854; moisture less than 0.003 per cent.; ash, 0.003 per cent.; reducing substances estimated as invert sugar, 0.002 per cent. This sugar is prepared by repeated crystallization. The Bureau of Standards states: "This material is the result of many years experimentation and experience in handling and in methods of preparation. The impurities have been reduced to the lowest possible point consistent with the issuing of a standard sample" (3). Spectroscopic examinations carried out in The Rockefeller Institute laboratories in New York by Dr. George I. Lavin indicate that the inorganic impurities are largely calcium with some magnesium. Traces of calcium or magnesium are, of course, without significance (11). While an ash content of 0.003 per cent. still represents a total ash impurity of 0.6 mg. per liter of nutrient, it does not appear feasible to push the purification further at present. This paper gives the results of experiments designed to compare the effectiveness of this highly purified sucrose with that of "C.P. grade" sucrose such as was used in all earlier studies, with commercial cane sugar available in grocery stores, and with Pfanstiehl's "C.P. grade" dextrose, considered by Robbins and Bartley (6) and by Robbins and Schmidt (9) as being equally effective with sucrose as a carbohydrate source for tomato roots.

EXPERIMENTATION

Roots of this laboratory's standard clone were used for these tests. They had been grown for 301 passages in the yeast nutrient earlier employed and then for three passages in a completely synthetic (glycine-thiamin) nutrient (17), which is now used as a standard in all work. The nutrients tested contained the standard salts and accessory salts discussed elsewhere (16), 3 p.p.m. glycine (17) and 0.1 p.p.m. thiamin (15), and carbohydrate. Twenty flasks contained the usual 2 per cent. Pfanstiehl "C.P. grade" sucrose and represented the standard control. Twenty contained 2 per cent. commercial cane sugar purchased in a local grocery, 20 contained 2 per cent. Bureau of Standards standard-sample sucrose as described above, 20 contained 2 per cent. Pfanstiehl "C.P. grade anhydrous" dextrose of the same make and grade as that used by Robbins and Bartley (6) and Robbins and Schmidt (9) and, since a 2 per cent. solution of dextrose has an osmotic value twice that of a 2 per cent. sucrose solution, a fifth set of 20 was prepared containing 1 per cent. dextrose, isotonic with the sucrose solutions. Each flask contained a single root, the inocula having been carefully selected to insure uniformity. Roots were grown in these five solutions through three consecutive passages and the increment rates, numbers of branches, and general appearance compared. The series was

repeated once. The results of these 600 cultures are shown in figures 1 and 2.

The numerical growth indices for the three samples of sucrose did not differ consistently or significantly either from sample to sample or from passage to passage in solutions containing the same sample, although the

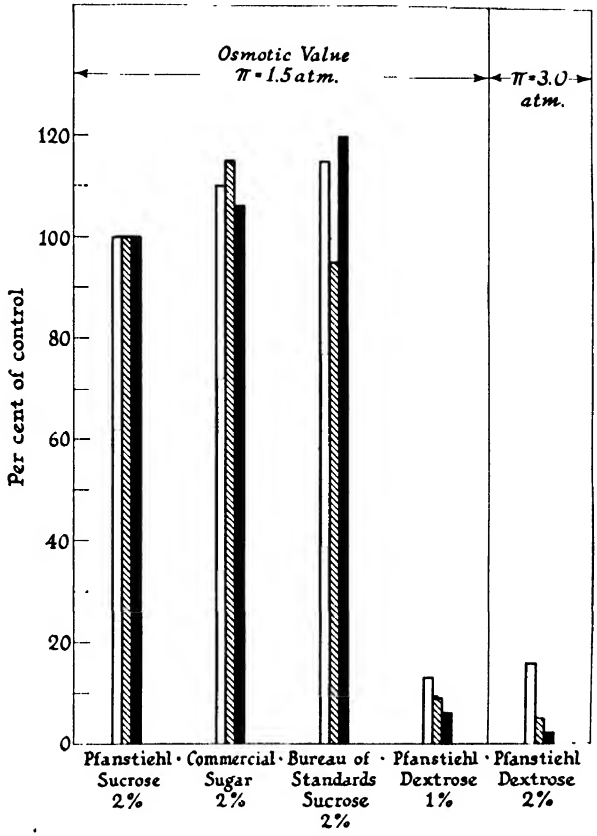

FIG. 1. Numerical indices of growth of excised tomato roots in various sugar solutions as indicated. Open columns represent the first passage, hachured columns the second passage, and solid columns the third passage in the experimental solution.

average index for store sugar and for Bureau of Standards sucrose was in each case about 10 per cent. greater than the control (18). Nor did the average number of branches formed differ significantly. The growth habits, however, did differ somewhat. Roots in Pfanstiehl's "C.P. grade" sucrose and in grocery store sugar were almost identical in appearance, being rather thick, white and bent. The average maximum length of their branches was 10 mm. and 9 mm., respectively. Those grown in the

Bureau of Standards sucrose were somewhat more slender and flexible, not bent, with somewhat shorter branches (7 mm. average maximum) but more regular habits. While experience has shown that, in general, thickened roots of the type formed in the first two sugars result from slightly injurious cultural conditions, the difference was not sufficiently marked to be of certain significance. Roots grown in dextrose solutions, on the other

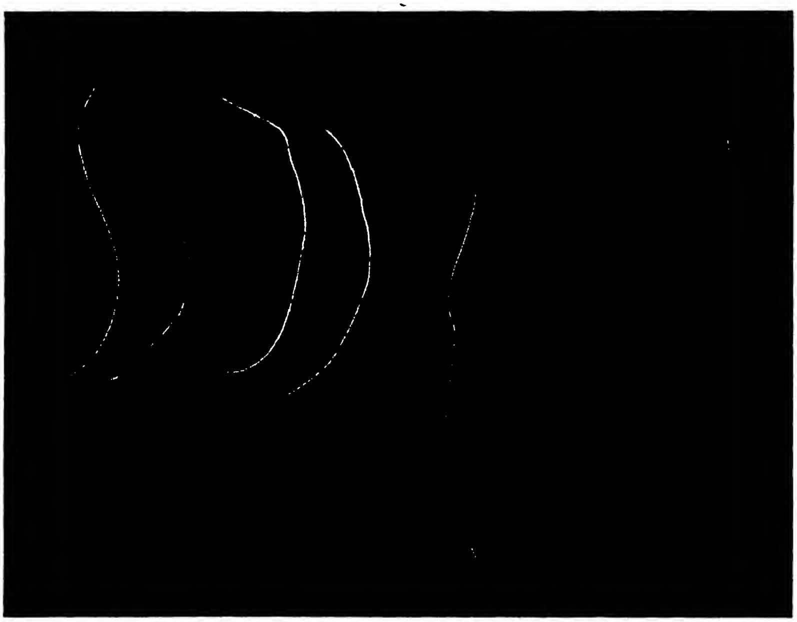

FIG. 2. The two roots at the left were grown in 2 per cent. store sugar, the set next to them in 2 per cent. Pfanstiehl "C. P. grade" sucrose, those in the middle in 2 per cent. Bureau of Standards sucrose, those next in 2 per cent. Pfanstiehl "C. P. grade" dextrose and those at the far right in 1 per cent. Pfanstiehl "C. P. grade" dextrose. The photograph was taken at the end of three passages in each solution. (Photograph by J. A. Carlile.)

hand, were definitely inferior to those grown in any of the sucrose solutions (18). This last finding is contrary to the conclusions of Robbins and Bartley (6) and Robbins and Schmidt (9) and supports the earlier conclusions of the writer (13).

DISCUSSION AND CONCLUSIONS

From the data presented here, it is clear that growth in the most highly purified sample of sucrose was slightly superior to that obtained in the less pure samples. Pfanstiehl's "C.P. grade" sucrose may contain impurities

which affect the growth of excised tomato roots, but these impurities certainly do not stimulate elongation. They tend rather to retard it. The Bureau of Standards sucrose contained about 35/1000 as much ash as did Pfanstiehl's "C.P. grade," yet supported slightly more satisfactory growth than did the latter. A differential of 35:1000 in known ash content had little effect on growth and that opposed to the effect expected from the theories cited above. Pfanstiehl's "C.P. grade" sucrose does not appear to be superior in this respect to the particular sample of grocery store sugar used in these tests, although no evidence has been collected as to the relative reliabilities of sugar from these two sources. The facts can be reconciled with the idea of significant impurities only if we assume that both pure and impure sugars contained the same impurities at concentrations giving the same effects. Either the postulated impurities were organic and were not fractionated by the recrystallization process, or else they were present in quantities greatly in excess of the optimum, yet were not toxic at these high concentrations. These are rather large assumptions to make without supporting evidence. And the facts are equally well in agreement with the alternative and much simpler assumption that significant impurities were not present in these materials.

It is, therefore, concluded that the best evidence at present available does not indicate the existence in "C.P. grade" sucrose of impurities necessary for, or clearly beneficial to, the growth of excised tomato roots.

LITERATURE CITED

1. Fiedler, H. Die pflanzliche Gewebe- und Organkultur. Zeitschr. Bot. **33**: 369–416. 1938.
2. Kotte, W. Kulturversuche mit isolierten Wurzelspitzen. Beitr. allg. Bot. **2**: 413–434. 1922.
3. National Bureau of Standards, Washington, D. C. Letter of February 13, 1939.
4. Robbins, W. J. Cultivation of excised root tips and stem tips under sterile conditions. Bot. Gaz. **73**: 376–390. 1922.
5. ———. Effect of autolized yeast and peptone on growth of excised corn root tips in the dark. Bot. Gaz. **74**: 59–79. 1922.
6. ———, and Bartley, Mary A. Use of dextrose by excised tomato roots. Science n.s. **86**: 290–291. 1937.
7. ———, and Maneval, W. E. Further experiments on growth of excised root tips under sterile conditions. Bot. Gaz. **76**: 274–287. 1923.
8. ———, and ———. Effect of light on growth of excised root tips under sterile conditions. Bot. Gaz. **78**: 424–432. 1924.
9. ———, and Schmidt, Mary Bartlett. Growth of excised roots of the tomato. Bot. Gaz. **99**: 671–728. 1938.
10. ———, and White, Virginia B. Limited growth and abnormalities in excised corn root tips. Bot. Gaz. **98**: 209–242. 1936.

11. White, P. R. Concentrations of inorganic ions as related to growth of excised root tips of wheat seedlings. Plant Physiol. **8**: 489–508. 1933.
12. ———. Potentially unlimited growth of excised tomato root tips in a liquid medium. Plant Physiol. **9**: 585–600. 1934.
13. ———. Plant tissue cultures. Bot. Rev. **2**: 419–437. 1936.
14. ———. Cultivation of excised roots of dicotyledonous plants. Amer. Jour. Bot. **25**: 348–356. 1938.
15. ———. Vitamin B_1 in the nutrition of excised tomato roots. Plant Physiol. **12**: 803–811. 1937.
16. ———. Accessory salts in the nutrition of excised tomato roots. Plant Physiol. **13**: 391–398. 1938.
17. ———. Glycine in the nutrition of excised tomato roots. Plant Physiol. **14**: 527–538. 1939.
18. ———. Sucrose *vs.* dextrose as carbohydrate source for excised tomato roots. Plant Physiol. **15**: 355–358. 1940.

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SUCROSE VS. DEXTROSE AS CARBOHYDRATE SOURCE FOR EXCISED TOMATO ROOTS

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In early studies on the cultivation of excised roots of wheat (3) carbohydrate was supplied in the form of "C. P. grade" dextrose (Pfanstiehl) at a concentration of 2 per cent. This choice was based on Robbins's earlier studies on roots of corn and was made without personal experimentation. Since the results seemed to be satisfactory, and interest at that time centered rather around other problems, no attempt was then made to determine the relative merits of other sugars. When, in 1932, attention was turned from wheat to tomato, comparably satisfactory results were not at first obtained. A survey of carbohydrate sources was made to determine if dextrose, shown to be satisfactory for wheat, might be surpassed by some other sugar. The carbohydrates tested were: one pentose (1-arabinose); five hexoses (d-mannose, d-galactose, d-glucose, d-fructose, and i-inosite); three di-saccharides (sucrose, lactose, maltose); one tri-saccharide (raffinose); one polysaccharide (dextrin); and one unidentified mixture (honey). All of these except the mannose and maltose (both Kahlbaum) and the honey were Pfanstiehl "C. P. grade" products. They were made up in nutrients containing the standard salts and yeast extract of the solution earlier developed and were introduced at concentrations equimolecular with the 2 per cent. dextrose solution previously employed. The results of that test are summarized in table I. They represent an unequivocal demonstration of the superiority of sucrose under these experimental conditions over all of the other sugars examined. A concentration of 2 per cent. sucrose was subsequently found somewhat superior to the 4 per cent. equimolecular with the 2 per cent. dextrose used for wheat. Since the major interest at that time was not to investigate carbohydrate metabolism but rather to find conditions which would permit unlimited growth, the details of these experiments were not published and the question was passed over with the statement (1934) that "sucrose . . . was found experimentally to be superior to dextrose (for tomato)" (4, p. 587) and (1936) that "tomato requires sucrose, being unable to utilize dextrose under the conditions studied" (5, pp. 430–431).

These statements stood unchallenged until 1937 and 1938 when Robbins and Bartley (1) and Robbins and Schmidt (2) concluded on the basis of their own experiments that "tomato roots *are* able to assimilate dextrose." These authors used several strains of tomato roots, including one obtained from the present writer, and used several sources of sucrose and dextrose. Their results do not show dextrose to be superior to sucrose but do show that carbohydrate source was not a limiting factor in their experiments (2, table 14). They are not directly comparable with results obtained in this laboratory, since a different source of yeast, a different length of culture period, and a different method of evaluating growth were employed. They are, nevertheless, sufficiently at variance with the results obtained here to make a re-examination of the question desirable. No attempt has been made to repeat the work of these authors. My own earlier experiments have been repeated using a technique similar to that which was employed before, except for minor improvements introduced from time to time during the past six years.

TABLE I

Growth of Excised Tomato Roots in Nutrients Containing Equimolecular Solutions of Various Sugars

(*Data Collected in December, 1932, and January, 1933*)

Sugar	Mean growth rate mm./cult./day	Condition
l-arabinose	None	Crooked, pale. No growth
d-mannose	0.3	Slender, white, bent. Good condition
d-galactose	0.3	Slender, brown, tips black. Poor condition
d-glucose	0.5	Swollen, brown, branched. Poor
d-fructose	0.5	" " " "
i-inosite	0.6	Slender, white, tips dead. Poor
sucrose	*3.6*	Slender, clean, white. Excellent
lactose	0.6	Slender, clean, white. Good condition
maltose	0.5	Slender, brown, tips black. Poor
raffinose	0.7	Slender, clean. Bases green. Good condition
dextrin	0.7	Slender, clean, white. Good condition
honey	None	Swollen, brown. Poor condition

The roots used were of this laboratory's "Standard Clone C," also tested by Robbins and Bartley (1) and Robbins and Schmidt (2), and were in the 304th passage at the beginning of the experiments. The control nutrient was the completely synthetic solution employed as standard since June 21, 1938. This nutrient regularly gives results identical with those obtained in the earlier yeast extract medium. It contains the six salts of a

modified Uspenski solution, four accessory salts, 0.1 p.p.m. thiamin and 3 p.p.m. glycine, and 2 per cent. Pfanstiehl "C. P. grade" sucrose. Its osmotic value is π = *ca.* 1.5 atm. Four solutions were compared with this, all identical except for the carbohydrate. These were made up with (1) 2 per cent. commercial sugar bought in a local grocery; (2) 2 per cent. Bureau

TABLE II

Relative Growth Rates of Excised Tomato Roots in Nutrients Containing Sugars of Various Sorts, Concentrations, and Degrees of Purity

Sugar	Increment as percentage of control			
	Passage			Average
	1	2	3	
Sucrose Pfanstiehl C. P. 2 per cent.	100	100	100	100
Sucrose Bureau of Standards 2 per cent.	115	95	120	110
Sucrose Commercial 2 per cent.	110	115	106	110
Dextrose Pfanstiehl C. P. 1 per cent.	13	9	6	9
Dextrose Pfanstiehl C. P. 2 per cent.	16	5	2	8

of Standards specially purified sucrose (6); (3) 2 per cent. Pfanstiehl "C. P. grade anhydrous" dextrose as nearly identical with that used by Robbins and Bartley and Robbins and Schmidt as obtainable, and giving an osmotic value of π = *ca.* 3.0 atm.; and (4) 1 per cent. Pfanstiehl "C. P. grade anhydrous" dextrose having an osmotic value the same as that of the sucrose solutions. Cultures were grown in the laboratory used for all of the writer's work of the past six years, in diffuse daylight. Twenty cultures were grown in each solution, the experiments were carried through three passages, and the entire series was repeated once. The results of these 600 cultures are presented in table II (see also 6, figs. 1 and 2). While growth in the three samples of sucrose was excellent and did not differ sig-

nificantly from the control nor diminish consistently from passage to passage, that in both concentrations of dextrose was extremely poor—less than 10 per cent. of that in the control nutrient—and it decreased in 2 per cent. dextrose from 16 per cent. in the first passage to 2 per cent. in the third, and in 1 per cent. dextrose from 13 per cent. in the first passage to 6 per cent. in the third.

The result of this experiment thus agrees with that obtained in 1933. Under the experimental conditions used as standard in this laboratory for the past six years, sucrose is superior to dextrose as a source of carbohydrate for excised tomato roots. Since the experiments were carried out with the same brand and quality of sugars and with the same strain of roots as were used by Robbins and Bartley (1) and Robbins and Schmidt (2), the discrepancy between the results of these authors and those here presented remains unexplained.

LITERATURE CITED

1. Robbins, W. J., and Bartley, Mary A. Use of dextrose by excised tomato roots. Science n.s. 86: 290-291. 1937.
2. ———, and Schmidt, M. B. Growth of excised roots of the tomato. Bot. Gaz. 99: 671-728. 1938.
3. White, P. R. Influence of some environmental conditions on the growth of excised root tips of wheat seedlings in liquid media. Plant Physiol. 7: 613-628. 1932.
4. ———. Potentially unlimited growth of excised tomato root tips in a liquid medium. Plant Physiol. 9: 585-600. 1934.
5. ———. Plant tissue cultures. Bot. Rev. 2: 419-437. 1936.
6. ———. Does "C. P. grade" sucrose contain impurities significant for the nutrition of excised tomato roots? Plant Physiol. 15: 349-354. 1940.

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SOLUBILITY STUDIES ON PURIFIED TOBACCO MOSAIC VIRUS

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Solubility studies on many crystalline proteins have shown that even large molecules show solubility behavior in general accord with that expected for homogeneous substances from the phase rule. Such studies in the case of the crystalline hemoglobins and enzymes have yielded much valuable information as to their identity and purity (1, 2). Solubility theory should apply also to particles larger than the simple proteins and experiments with a bacteriophage by Northrop indicated that this material, although having a minimum molecular weight of about 500,000, possessed a fairly definite solubility (3). It has been shown that tobacco mosaic virus purified by centrifugation or by careful treatment with ammonium sulfate is homogeneous when examined either by sedimentation in the ultracentrifuge or by electrophoresis in the Tiselius apparatus (4, 5). These methods, however, show only that all of the material in the sample sediments in a centrifugal field or migrates in an electrical field at uniform rates. Although many other experiments also failed to provide evidence for inhomogeneity (6), it appeared desirable to study the solubility behavior of the virus. In the experiments to be described in this paper, solubility determinations were made on tobacco mosaic virus purified by different methods (7, 8), on samples isolated at varying intervals of time after inoculation (9), and on samples isolated from different host plants, in an effort to determine whether purified virus behaves as a homogeneous material from a solubility standpoint.

Materials and Methods

The virus samples were obtained by the more recent chemical procedures which involved only a few precipitations with ammonium sulfate and by ultracentrifugation with or without a preliminary precipitation with ammonium sulfate. When purified by the earlier procedures, the virus was recrystallized several times before it was used. When prepared by ultracentrifugation it was used without a preliminary crystallization other than that involved in the solubility determination. Samples prepared by ammonium sulfate precipitation were dialyzed on a rocking dialyzer (10) against distilled water until they failed to give a test for ammonia with Nessler's solution. The samples

prepared by ultracentrifugation were obtained by the sedimentation of the virus in the clarified juice or in concentrates prepared by precipitation of the virus with ammonium sulfate and re-solution in water. The virus pellets which separated during the ultracentrifugation were redissolved either in 0.1 M potassium phosphate buffer at pH 7 or in water and resedimented one or two additional times. The samples taken from plants 2 to 13 weeks after inoculation were portions of preparations isolated and described by Stanley (9) and used for sedimentation analyses by Wyckoff (5). Solutions in 0.1 M phosphate containing 2 to 3 per cent virus were stored at 4° for from 6 to 12 weeks before the solubility determinations were made.

Preliminary experiments showed that centrifugation in an angle head in an ordinary laboratory centrifuge served much better than filtration during a solubility determination to separate suspended crystals of virus from that in solution. Equilibrium was approached from the supersaturated side by adding to the sample of virus dissolved in 0.1 M phosphate buffer a weighed amount of ammonium sulfate sufficient to cause the crystallization of a part of the virus in solution. Equilibrium was approached from the undersaturated side either by crystallizing the virus at the isoelectric point with dilute phosphoric acid and extracting the sedimented virus, after the supernatant fluid had been thoroughly drained, with buffer containing ammonium sulfate (method 1); or by adding solid ammonium sulfate to the sample dissolved in a sufficiently small volume of buffer to cause the separation of all the virus, and then adding sufficient buffer to reduce the salt concentration to the desired level (method 2). In a few instances, solubilities were determined at room temperature. When this was done and the samples were centrifuged at 3000-4000 R.P.M. for 3 to 4 hours, the virus concentration in the supernatant liquid decreased only slightly after additional centrifugation. In most experiments, however, the virus suspensions, cooled to 0°, and stirred for 10 to 15 minutes, were allowed to stand for 24 hours at 4° and then were centrifuged at 3000-4000 R.P.M. for about 2 hours at the same temperature. The supernatant liquid was pipetted off and analyzed for protein nitrogen as previously described (7). The crystals which separated under these conditions, while very small in size, were the typical needle-like crystals. Because of the extremely small size of the crystals, it is difficult to be certain that amorphous material was absent in all cases. However microscopic examination did not show material of a definitely amorphous character.

RESULTS

Examples of the results obtained with virus samples purified by chemical procedures and by sedimentation in the ultracentrifuge before and after preliminary precipitation with ammonium sulfate, and the effect of storage in 0.1 M phosphate buffer or 20 per cent ammonium sulfate are shown in Table I. Samples purified by chemical procedures varied in solubility at room temperature in 5.25 per cent ammonium sulfate and 0.1 M phosphate at pH 5.6 from 0.26 to 1.5 mg. virus N per ml. Samples prepared by ultracentrifugation showed a similar variation. Some, at the concentrations tested, were entirely soluble in a concentration of ammonium sulfate at 4° which, for another sample, gave a solubility of about 0.3 mg. N per ml. at the same temperature. The solubility of a sample of virus varied greatly

with the concentration of ammonium sulfate used as solvent. A sample which was soluble to the extent of 0.93 mg. N per ml. in 11.18 per cent ammonium sulfate gave a solubility of 0.07 mg. N per ml. in 11.77 per cent ammonium sulfate. Storage for a month at 4° in 0.1 M phosphate buffer

TABLE I

Solubilities in Ammonium Sulfate and 0.1 M Potassium Phosphate Buffer of Tobacco Mosaic Virus Prepared by Different Methods

Experiment	Method of preparation	Mg. virus N per ml. suspension	Mg. virus N per ml. solution
1	Precipitation with ammonium sulfate (4). Recrystallized 5 times. Isolated 3 weeks after inoculation. Solubility at room temperature in 5.25 per cent ammonium sulfate at pH 5.6 from supersaturated side	1.58	0.26, 0.26
2	Prepared and solubility determined as in 1. Plants inoculated with virus obtained from a single lesion	1.58	0.36
3 *a*	Prepared and determined as in 1. Recrystallized several times	1.58	0.94
b	The same sample after standing 1 month at 4° in 0.1 M phosphate buffer at pH 7	1.58	0.21
4 *a*	Prepared and determined as in 1. Solubility determined without preliminary crystallization	1.58	1.50
b	The same sample after standing as in 3*b*		0.21
5	Prepared and determined as in 1. Solubility determined about a year after standing at 4° as a paste in 20 per cent ammonium sulfate	3.16	0.26
6 *a*	2 sedimentations in ultracentrifuge. Solubility at 4° in 11.18 per cent ammonium sulfate at pH 5.6 from supersaturated side	0.93	0.93
b	Same as 6*a*. Solubility in 11.77 per cent ammonium sulfate	0 93	0.07
7	Prepared and determined as in 6*a* in 11.77 per cent ammonium sulfate	0.93	0.05
8 *a*	3 sedimentations in ultracentrifuge. Solubility as in 6*a* at pH 6.4	0.76	0.25, 0.21
b	Same as 8*a*. From undersaturated side (method 2)	0.76	0.26, 0.26
c	Same as 8*b*	1.00	0.25, 0.26
d	Precipitated once with ammonium sulfate and purified by 3 sedimentations in ultracentrifuge. Solubility as in 8*b*	1.00	0.38, 0.35

or for about a year in 20 per cent ammonium sulfate reduced the solubility from relatively high values at room temperature to about 0.1–0.2 mg. N per ml. and repeated recrystallization at room temperature seemed to produce a similar result. Evidence has been obtained that these treatments cause an irreversible linear aggregation of the virus particles (11, 12). One precipitation with ammonium sulfate at 4°, which does not cause a measurable amount of such aggregation, also failed to cause any pronounced

change in the solubility of the virus. The specific activities of the original samples of virus used in the experiments given in Table I were not deter-

TABLE II

Solubilities at 4° in 0.1 M Potassium Phosphate Buffer at pH 5.6 and 11.18 Per Cent Ammonium Sulfate of Tobacco Mosaic Virus Isolated from Plants Harvested from 2 to 13 Weeks after Inoculation

No.	Weeks after inoculation isolated	Method of preparation*	Mg. virus N per ml. solution†
1 *a*	2	Two sedimentations in ultracentrifuge. Solubility determined from supersaturated side	0.36
b		The same sample after standing 1 month longer at 4° in 0.1 M phosphate buffer at pH 7	0.54
2 *a*	3	Prepared and determined as in 1*a*	0.52
b		Precipitated once with ammonium sulfate and purified by 2 sedimentations in ultracentrifuge. Solubility as in 1*a*	0.14, 0.15
3 *a*	4	Prepared and determined as in 1*a*	0.19, 0.19
b		The same sample after standing as in 1*b*	0.08
4 *a*	5	Prepared and determined as in 1*a*	0.48
b		The same sample after standing as in 1*b*	0.17, 0.12
c		Prepared and determined as in 2*b*	0.02
5	6	Prepared and determined as in 1*a*	0.40, 0.62
6 *a*	7	Prepared and determined as in 1*a*	0.61, 0.48
b		The same sample after standing as in 1*b*	0.27
7 *a*	8	Prepared and determined as in 1*a*	0.50
b		The same sample. Solubility determined from undersaturated side (method 1)	0.48, 0.48
c		The same sample after standing as in 1*a*	0.40
d		Prepared and determined as in 2*b*	0.30
8	13	Prepared and determined as in 1*a*	0.77, 0.82

* The samples were stored in 0.1 M phosphate solution at 4° for from 6 to 12 weeks before the solubility determinations were made.

† Solubility determinations were made in all cases on 0.93 mg. virus N per ml. suspension.

mined, but they were prepared by comparable procedures involving a minimum of treatment with ammonium sulfate or exposure to room temperature. In previous experiments it has been shown that such samples possessed comparable specific activities and gave sharp boundaries in the analytical ultracentrifuge.

The solubility experiments with the samples isolated by Stanley (9) from plants 2 to 13 weeks after inoculation were carried out from 6 to 12 weeks later. Those isolated from plants 2 to 8 weeks after inoculation gave relatively constant results (Table II). The mean value for six samples in ten determinations was 0.50 ± 0.02 mg. virus N per ml. at 4° in 11.18 per cent ammonium sulfate and 0.1 M phosphate at pH 5.6. The solubility of the 13 week sample, however, was about 0.8 mg. N per ml. under the same conditions. Virus that had been precipitated once in the cold with ammonium sulfate and then ultracentrifuged showed, in general, a decrease in solubility as compared with virus prepared from the same sap by ultra-

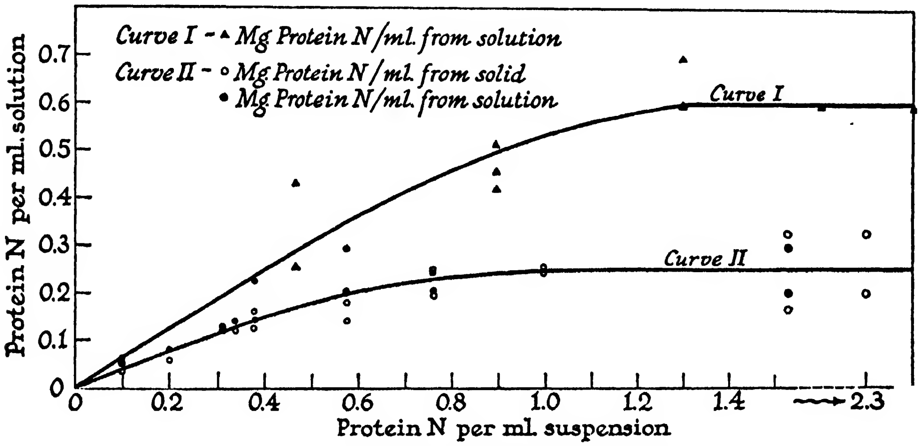

FIG. 1. Solubility of two ultracentrifuged preparations of tobacco mosaic virus in 0.1 M potassium phosphate and 11.18 per cent ammonium sulfate buffer: curve I at pH 5.6, curve II at pH 6.4.

centrifugation. A good deal of variation was found, however. Some samples were entirely insoluble after the ammonium sulfate treatment while others were nearly as soluble as the ultracentrifuged virus. The variation was probably due to differences in the length of time different samples had been allowed to stand in ammonium sulfate solution during the isolation procedure. Similarly, after standing for about a month longer in 0.1 M phosphate at 4°, the solubility of most of the samples tested decreased further.

The solubility data obtained with varying amounts of solid phase in the presence of a constant volume of solvent showed an increase in the amount of dissolved virus as the amount of solid phase was increased above that amount necessary to give a saturated solution. The results with two preparations obtained by ultracentrifugation are shown graphically in Fig. 1.

The data are not sufficiently precise to establish definitely the shape of the solubility curves. Relatively constant solubilities were obtained, however, as the amount of virus in suspension was increased beyond 1.3 mg. N per ml. in one case and beyond 1 mg. N per ml. in the other, but in both instances some solid phase separated in the presence of much smaller amounts of virus. In the case examined most completely (curve II) some solid virus separated or failed to dissolve even at one-tenth the concentration which seemed to give a constant solubility. Similar results were obtained with a sample purified by precipitation with ammonium sulfate. The solubility curves are not characteristic of a material consisting of only one solid phase but resemble those of solid solutions (13). A similar conclusion was reached when a crystallized sample of virus was extracted repeatedly with equal portions of the same solvent, for the amount of dissolved virus decreased progressively as the amount of virus used as saturating body decreased.

A possible explanation for the presence of solid phase at the lower virus concentrations was the formation of a fraction consisting of relatively insoluble and inactive virus as a result of the contact with the ammonium sulfate solution during the solubility determination. If this were the correct explanation then the specific activity of the crystallized virus would be expected to be lower than that of the virus in solution. To test this hypothesis the specific activities of the crystallized virus and the virus in solution from two solubility determinations (curve II, Fig. 1) in which the original virus concentrations were 0.38 and 0.57 mg. virus nitrogen per ml., respectively, were compared. The crystallized virus was dissolved in water in each case and these solutions and those containing the soluble virus were dialyzed until free of ammonium sulfate and analyzed for nitrogen. The dialyzed solutions of crystallized and soluble virus in one experiment were diluted to 10^{-5} gm. virus per ml. with 0.1 M phosphate buffer at pH 7 and compared on 42 half leaves of *Nicotiana glutinosa*. In two tests, 2392 and 2904 lesions were obtained for the solution of crystallized virus as compared with 2002 and 2602 lesions, respectively, for the virus in solution. In the second experiment, the dialyzed solutions were diluted to 2×10^{-6} gm. virus per ml. and compared on 37 half leaves of *Phaseolus vulgaris* L. var. Early Golden Cluster. In two tests, 253 and 627 lesions were obtained for the crystallized virus as compared with 388 and 508, respectively, for the virus in solution. When examined statistically on the basis of half-leaf units by "Student's" method (14), the crystallized virus in two tests was significantly more active than that in solution, the virus in solution was significantly more active in one test and in the other

the difference in lesion counts was not significant. When the total numbers of lesions obtained by each treatment were combined and evaluated similarly, the crystallized virus rather than that in solution proved significantly more active. It may be concluded, therefore, that the fraction of virus which precipitated did not consist of inactive or largely inactive virus.

The question of a change in tobacco mosaic virus as a result of treatment with ammonium sulfate was also studied by comparing the activities of samples of virus isolated from portions of the same infected sap by ultracentrifugation and by ammonium sulfate precipitation in the cold. In eight tests in which a total of 305 half leaves of *Phaseolus vulgaris* was used for the comparison of samples isolated from time to time, a total of

TABLE III

Solubility Experiments with Tobacco Mosaic Virus from Tobacco and Tomato Plants

	Mg virus per ml. supernatant after centrifugation for 2 hrs. at about 4°C.
(1) 50 mg. virus from tobacco plants in 10 cc. 0.1 M phosphate at pH 7 crystallized with 975 mg. $(NH_4)_2SO_4$	4.39
(2) 50 mg. virus from tomato plants in 10 cc. 0.1 M phosphate at pH 7 crystallized as in (1)	4.49
(3) 50 mg. crystals of virus from tobacco plants extracted with 10 cc. 0.1 M phosphate at pH 7 + 975 mg. $(NH_4)_2SO_4$	4.04
(4) 50 mg. crystals of virus from tomato plants extracted as in (3)	4.15
(5) 5 cc. of supernatant from (3) used to extract crystals from (4)	4.11
(6) 5 cc. of supernatant from (4) used to extract crystals from (3)	4.09

11,160 lesions was obtained for the samples prepared by ultracentrifugation as compared with 11,202 for the samples prepared by precipitation with ammonium sulfate. If the treatment with ammonium sulfate resulted in an irreversible change in virus in these experiments, it was not accompanied by a significant loss of virus activity. It should be noted, however, that a decrease in specific activity or a loss of active virus corresponding to less than about 10 per cent of the original might not be detected by the infectivity tests.

Solubility of Virus from Different Host Plants.—Preliminary experiments in which the solubilities of tobacco mosaic virus isolated from Turkish tobacco and tomato plants by chemical methods were compared, showed that about the same solubility behavior was obtained for virus isolated from either host provided the samples were isolated by the same procedures from plants inoculated for the same periods of time (7). Similar experi-

ments were carried out on ultracentrifuged preparations of virus from tobacco and tomato plants. The juice was obtained from young plants about a month after inoculation and the virus was precipitated once in the cold with 20 per cent ammonium sulfate. It was redissolved in 0.1 M phosphate buffer and sedimented twice in the ultracentrifuge. The experiments on two samples obtained in this manner are summarized in Table III. It may be seen that under the conditions used, about the same solubility was obtained for the tomato as for the tobacco sample when the solubility was approached either from the supersaturated or the undersaturated side. When the saturated solution obtained from the tobacco sample was used to extract the crystals which separated from the tomato sample, a slight decrease rather than increase in the amount of dissolved virus took place, and a similar result was obtained when the saturated solution from the tomato sample was used to extract the crystals which separated from the sample isolated from tobacco plants.

DISCUSSION

The wide variation found for the solubility of different samples of purified tobacco mosaic virus prepared by precipitation with ammonium sulfate and by ultracentrifugation, and the type of solubility curve obtained in the presence of varying amounts of solid phase indicate, contrary to the results of ultracentrifugal and electrophoretic analyses, that the purified virus is not a homogeneous material. The results are not readily explained either by the presence of inactive impurities, by the reversible or irreversible formation of inactive virus, or by failure to attain equilibrium during the solubility determinations. They may be explained by variations produced in the virus itself either during growth under different conditions or during isolation. Evidence for a small amount of variation in tobacco mosaic virus has been obtained previously (15) by the isolation of mutant strains from plants infected with a single infectious unit of the ordinary strain. These were recognized because of characteristic signs of disease on infected plants different from those of ordinary tobacco mosaic and have been carried serially through many generations of plants. The number and amount of such strains which are formed during the production of the virus in the host plants are not believed to be sufficient to account for the type of solubility curve found. In view of the fact that the more soluble and the less soluble fractions are equally infectious and, in the limited number of tests made, produce typical tobacco mosaic disease, it seems more likely that the solubility of the infectious agent itself may vary. It is possible that the less soluble virus may be derived from the

more soluble by a process similar to that taking place when the virus ages *in vitro*, or it may be that during the multiplication of the original infective unit other units differing slightly in solubility but causing typical tobacco mosaic are produced. The solubility data provide evidence that the purified virus is better described, as has already been indicated (16), as a family of closely related substances rather than as a single chemical individual. The experiments with the virus from tobacco and tomato plants show, however, that remarkably comparable virus may be obtained from plants of different genera when the latter are grown under the same conditions and the virus is isolated by similar procedures.

SUMMARY

Different samples of purified tobacco mosaic virus show a relatively wide variation in solubility in ammonium sulfate solution. This variation and the type of solubility curve obtained in the presence of varying amounts of solid phase show that the purified virus whether isolated by mild treatment with ammonium sulfate or by ultracentrifugation is not a homogeneous chemical substance but contains more soluble and less soluble virus fractions of comparable specific activities. Long contact with strong ammonium sulfate solutions or 0.1 M phosphate buffer results in a decrease in solubility. The variation in the solubility of samples isolated from different plants by the same method seems to depend in part on the length of time the plants are inoculated before they are cut, and probably also on the conditions under which they are grown. Virus preparations isolated from plants of different genera grown under the same conditions and inoculated at the same time, however, behaved like identical substances in solubility experiments.

BIBLIOGRAPHY

1. Landsteiner, K., and Heidelberger, M., *J. Gen. Physiol.*, 1930, **13**, 781.
2. Northrop, J. H., Crystalline enzymes. The chemistry of pepsin, trypsin, and bacteriophage. Columbia Biological Series, No. 12, New York, Columbia University Press, 1939.
3. Northrop, J. H., *J. Gen. Physiol.*, 1938, **21**, 335.
4. Eriksson-Quensel, I., and Svedberg, T., *J. Am. Chem. Soc.*, 1936, **58**, 1863.
5. Wyckoff, R. W. G., *J. Biol. Chem.*, 1937, **121**, 219.
6. Stanley, W. M., and Loring, H. S., Properties of virus proteins, in Cold Spring Harbor symposia on quantitative biology, Cold Spring Harbor, Long Island Biological Association, 1938, **6**, 341.
7. Loring, H. S., and Stanley, W. M., *J. Biol. Chem.*, 1937, **117**, 733.
8. Stanley, W. M., and Wyckoff, R. W. G., *Science*, 1937, **85**, 181.
9. Stanley, W. M., *J. Biol. Chem.*, 1937, **121**, 205.

10. Kunitz, M., and Simms, H. S., *J. Gen. Physiol.*, 1928, **11**, 641.
11. Loring, H. S., Lauffer, M. A., and Stanley, W. M., *Nature*, 1938, **142**, 841.
12. Lauffer, M. A., and Stanley, W. M., *Chem. Rev.*, 1939, **24**, 303.
13. Northrop, J. H., and Kunitz, M., *J. Gen. Physiol.*, 1930, **13**, 781.
14. Fisher, R. A., Statistical methods for research workers, Edinburgh and London, Oliver and Boyd, 6th edition, 1936.
15. Jensen, J. H., *Phytopathology*, 1933, **23**, 964; 1936, **26**, 266.
16. Stanley, W. M., Biochemistry and biophysics of viruses, in Doerr, R., and Hallauer, C., Handbuch der Virusforschung, Erste Hälfte, Vienna, Julius Springer, 1938, 520.

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